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A comparison of *Arcobacter butzleri* ED-1 and *Arcobacter* L anode biofilm formation and a proteomic comparison of *A. butzleri* ED-1 at the anode of a half microbial fuel cell

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Abstract

Microbial fuel cells (MFCs) are electrochemical devices that exploit the ability of certain microorganisms to anaerobically respire using an insoluble terminal electron acceptor and therefore generate an electrical current. These bacteria are called electrogens or electrogenic bacteria. Two species of *Arcobacter*, *Arcobacter butzleri* ED-1 and *Arcobacter* L were isolated from the anodic chamber of an acetate fed MFC, and *A. butzleri* ED-1 was found to be the more electrogenic of the two bacteria. *Arcobacter spp*. are ε proteobacteria and *A. butzleri* ED-1 and *Arcobacter* L were the first example of electrogenically active e proteobacteria. It was decided to study their interactions with the anode by fluorescent microscopy and study their electrogenic mechanisms by comparative proteomics using the iTRAQ method as it would allow for simultaneous identification and quantification of peptides in multiple samples. Fluorescent imaging over a period of 120 h in a half MFC showed that both *A. butzleri* ED-1 and *Arcobacter* L formed a thin anodic biofilm of a few cells thick and that *A. butzleri* ED-1 maintained a more stable anodic biofilm than *Arcobacter* L. iTRAQ analysis showed that the flagellin FlaA was up-regulated 2.4 fold at the anode but no other electron transport proteins or adhesins were up-regulated. These results were distinct from those observed for other electrogenic bacteria (*Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1) in previous studies which exhibited up-regulated electron transport proteins at the anode as well as forming an anodic biofilm of 50 μm thick. Therefore based on these results it was concluded that FlaA was most likely playing an important role *A. butzleri* ED-1

anode biofilm formation and that the mechanisms of electrogenesis in *A. butzleri* ED-1 and *Arcobacter* L may be novel compared to those previously characterised. It was also concluded that one possible reason for *A. butzleri* ED-1 being more electrogenic than *Arcobacter* L was its ability to form a more stable anodic biofilm. It must be noted that both of these conclusions are highly speculative and further study is needed to elucidate the electrogenic mechanisms of *A. butzleri* ED-1 and to further compare biofilm formation between the two species.

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Abbreviations

- **A_{600}-** absorbance measured at 600 nm
- **AB**-amino acid biosynthesis
- **AMM**- *Arcobacter* minimal media
- **BLAST**- Basic local alignment search tool
- **BOC**- British oxygen company
- **BUG**- benthic unattended generator
- **CA**- cell adhesion
- **cfu**- colony forming units
- **CM**- cytoplasmic membrane
- **CMM**- *Campylobacter* minimal media
- **CoA**- Co-enzyme A
- **COD**- chemical oxygen demand
- ***ColE1***- origin of replication from the ColE1 plasmid
- **CoTMPP**- cobalt tetramethoxyphenylporphyrin
- **CFP**- cyan fluorescent protein
- **CYT**- cytoplasmic
- **DGGE-** denaturing gradient gel electrophoresis
- **DMSO**- Dimethyl sulphoxide
- **dNTP**- deoxyribonucelotide
- **EC**- extracellular
- **EM**- electron microscopy
- **EPS**- exopolyscahhride
- **FA**- fatty acid metabolism
- **FePc**- iron (II) phthalocyanine
- **GFP**- green fluorescent protein
- **Gm^R**- gentamycin resistance cassette
- **HPLC**- high performance liquid chromatography
- **HY**- hypothetical
- **IM**- intermediary metabolism
- **iTRAQ**- isobaric tag for relative and absolute quantification
- **Km^R**- kanamycin resistance cassette
- **LC**- liquid chromatography
- **MC**-motility and chemotaxis
- **MCS**- multiple cloning sight
- **MFC**- microbial fuel cell
- **MQ**-Menaquinone
- **MS**- mass spectrometry

- **NADH**- Nicotinamide adenine dinucleotide
- **NEB**- new England biolabs
- **NU**- nucleaic acid metabolsim
- **OM**- outer membrane
- ***oriT***- origin of transfer
- **PBS**- phosphate buffered saline
- **PCR**- polymerase chain reaction
- **PH**- protein folding
- **PP**- periplasmic
- ***repB***- origin of replication from a 2 kb *Arcobacter butzleri* plasmid
- **RS**- response to stress
- **SEM**- scanning electron microscopy
- **SOC**- Super optimal broth
- **SOX**- sulphur oxidase system
- **TB**- transport proteins
- **TCA cycle**- tricarboxylic acid/ Krebs cycle
- **TEA**- terminal electron acceptor
- **TEM**- transmission electron microscopy
- **TF**- transferase
- **TL**- translation
- **TL(b)**- translation and localisation
- **TMAO**- Trimethylamine N oxide
- **UN**- unknown
- **XIC**- extracted ion chromatogram

Declaration

I declare that this thesis has been composed by myself, and that the work is my own unless otherwise stated; significant contributions from research groups I have been a part of are clearly indicated. This work has not been submitted for any other degree or professional qualification.

Signed……..

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Chapter 1

Introduction

This project focuses on the electrogenic ability (the ability to generate electricity) of *Arcobacter butzleri* ED-1 in a microbial fuel cell (MFC). An MFC is a device that exploits the ability of certain bacteria to respire anaerobically using an insoluble terminal electron acceptor to generate an electrical current from a variety of different organic fuel sources (Logan 2008). The ability of micro-organisms to generate electricity was first observed in 1911, where electrical effect accompanying the decomposition of organic compounds was first observed (Potter 1911). From the 1960s to 2004 MFCs were thought to require the addition of exogenous mediators to generate a reasonable amount of power and thus were not considered for practical applications (Logan and Reagen 2006). However the discovery of microbiological consortia capable of generating reasonable amounts of electricity (these systems generated currents of up to 0.2 mA) without exogenous mediators shed new light on the field and suggested it could be employed for more practical purposes (Kim *et al*., 1999, Gil *et al*., 2003, Kim *et al*., 2004, Tender et *al*., 2002). MFCs will be discussed in more detail in section 1.2. However in order to understand and MFCs in any detail the basic principles of fuel cells in general must first be understood.

1.1 Fuel cells

A fuel cell is a device that converts the chemical energy of fuel directly into electrical energy. Fuel cells have been developed for use with a variety of different fuels, the most common being hydrogen (Vishnyakov 2006). Hydrogen fuel cells will be used to illustrate the electrochemical reactions in a fuel cell.

1.1.1 The electrochemical reactions of hydrogen fuel cells

In a hydrogen fuel cell, H_2 is oxidised to H^+ and e^- at the anode via a catalyst such as platinum. H^+ then migrates through a partially permeable membrane to the cathode where H^+ is reduced to H_2O (Vishnyakov 2006). The difference in redox potential between the H^+ and O^- results in a generation of current, with a typical fuel cell generating voltages of 0.4 V- 0.7V although they can be stacked to generated higher voltages (Palma and Enjeti 2009, Palma *et al*., 2009). Figure 1.1 illustrates the reactions occurring in a hydrogen fuel cell.

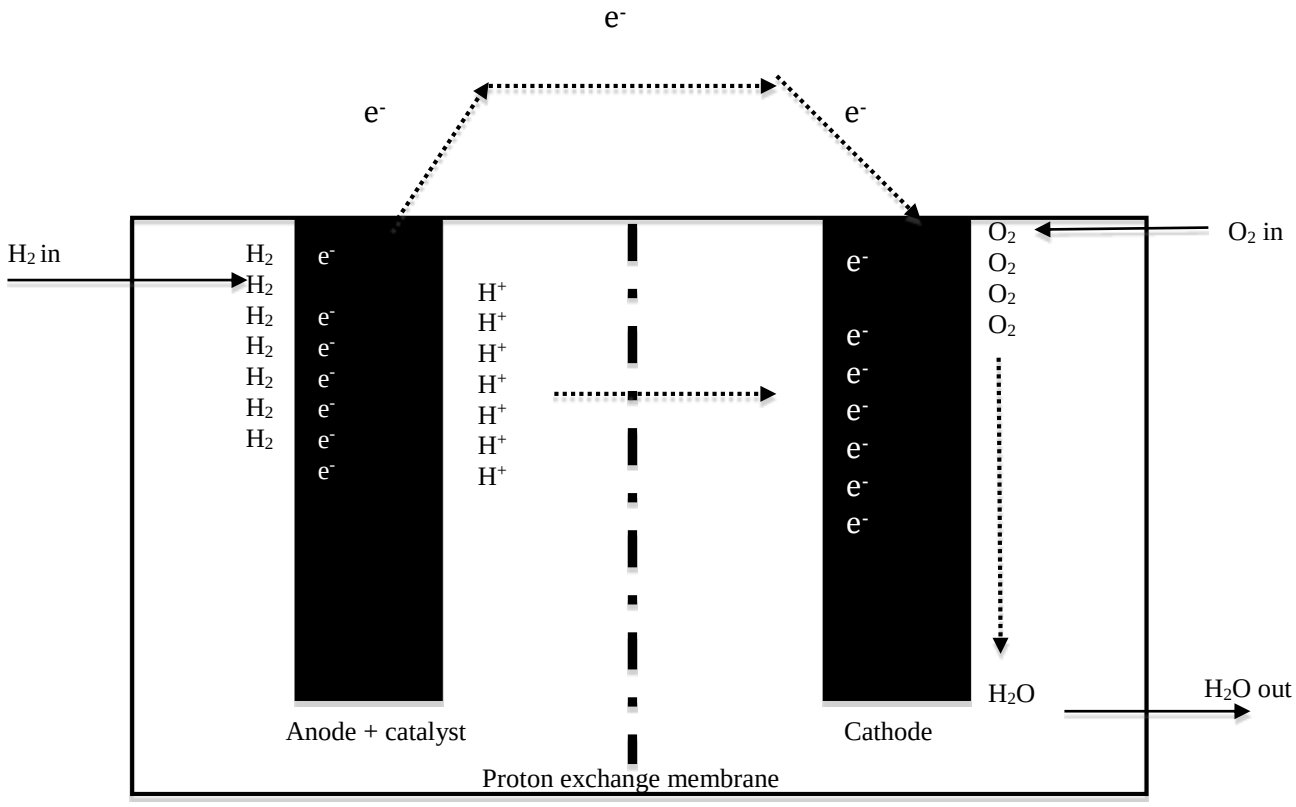


Figure 1.1 Figure 1.1 shows a schematic diagram of a typical hydrogen fuel cell. H_2 flows into the cell where it is oxidised at the anode ($2H_2 \rightarrow 4H^+ + e^-$). The protons and electrons then migrate to the cathode (with protons crossing over a proton exchange membrane) where oxygen is reduced to water ($O_2+4H^+ \rightarrow 2H_2O$). This series of redox reactions (overall reaction $2H_2+O_2 \rightarrow 2H_20$) generates electricity of 0.6-0.7 V.

As can be seen in Figure 1.1 a hydrogen fuel cell generates electrical energy directly from the oxidation of H_2. There are a number of advantages to using Hydrogen fuel cells over fossil fuels.

1.1.1 The advantages of fuel cell use

Fuel cells produce zero emissions if using H_2 and only a small amount of emissions if using natural gas (Cheng *et al*., 2007). Low emissions mean that fuel cells generate a lot less pollution than fossil fuels and it is predicted that replacing the current fossil fuel system of energy supply then atmospheric pollution would be reduced by up to 90% (Grube and Stolten, 2010). Fuel cells do not produce much noise pollution with a typical fuel cell generating around 60 dBa (Haile, 2003). Fuel cells typically operate at 40-60 % energy efficient, and this can be improved to approximately 80 % if the heat lost from a fuel is used to heat a location (www1.eere.energy.gov 2012). The exact energy efficiency of a fuel cell depends upon the specifics of its design (www.1.eere.energy.gov 2012). However in general fuel cells are more efficient than coal fired power stations which are typically 33 % energy efficient (www.1.eere energy.gov 2012).

Natural gas, most typically methane can be used in a fuel cell set-up which directly oxidises the hydrocarbon (Murray and Barnett 1999). Other hydrocarbons used in fuel cells include ethane, *n* butane, 1-burene and toluene (Park *et al*., 2000). This requires a set-up known as a solid oxide fuel cell, which is a set-up which uses a ceramic anode (Murray and Barnett, 1999). One advantage of the solid oxide fuel cell is that it allows for a greater range of fuel sources to be used which would bypass the costly requirements for setting up a H_2 infrastructure (Bossche and McIntosh 2013). However the Nickel-ceramic anodes used in solid oxide fuel cells can react with dry hydrocarbons to produce graphite, which deposits on the anode

inhibiting their performance. This can be counteracted by supplying large amounts of steam, which increases operating costs (Bossche and McIntosh 2013).

The efficient and green nature of fuel cells means they have seen use in a number of different situations.

1.1.2 The uses of fuel cells

Fuel cells have been used to supply power in a number of different commercial, industrial and residential applications and a number of prominent companies have used fuel cell technology to supply, in part, their power needs (Grasman 2013). The reliability of fuel cells means they are useful as a power source in remote locations such as spacecraft, remote weather stations and communications centres (Grasman 2013). In addition the carbon- neutral nature of fuel cells makes them of considerable interest as a power source for vehicles.

A number of prototype automobiles powered by hydrogen fuel cells have been developed (Williams and Kurani, 2007). Hydrogen powered automobiles and other vehicles represent a potential solution to the current environmental problems such as CO_2 emissions caused by the use of Fossil fuels to power vehicles as hydrogen powered vehicles produce zero emissions from the tailpipe (Williams and Kurani, 2007; Offer *et al*., 2010). Therefore hydrogen fuel cells are theoretically a viable alternative to fossil fuels although they are currently limited by problems with

storage; hydrogen at room temperature it takes up an impractically large amount of space e.g. at room temperature 5 kg of hydrogen occupies 56,000 L and therefore requires compression to be of use in vehicles (Schlapbach, 2009).

Hydrogen, methanol and methane based fuel cells are not the only type of fuel cell; another type of fuel cells are MFCs which exploit the electrochemical properties of certain bacteria to generate electricity from a wide range of fuel sources.

1.2 Microbial fuel cells

Like all fuel cells, MFCs directly convert the chemical energy of fuel into electricity (Logan 2008). However unlike the fuel cells discussed in section 1.1 MFCs use electrochemically active microorganisms growing on the anode/ within the anode chamber to oxidise the fuel to H^+ and e^- i.e. in a MFC microbes function as a biocatalyst for the oxidation of fuel (Bond and Lovely 2003). In brief MFCs exploit the ability of certain bacteria to use an insoluble terminal electron acceptor (TEA) during anaerobic respiration, which is a common physiological trait of anaerobic metal reducing bacteria. Extracellular electron transfer is discussed in detail in section 1.6. Figure 1.2 gives a schematic representation of how MFCs work.

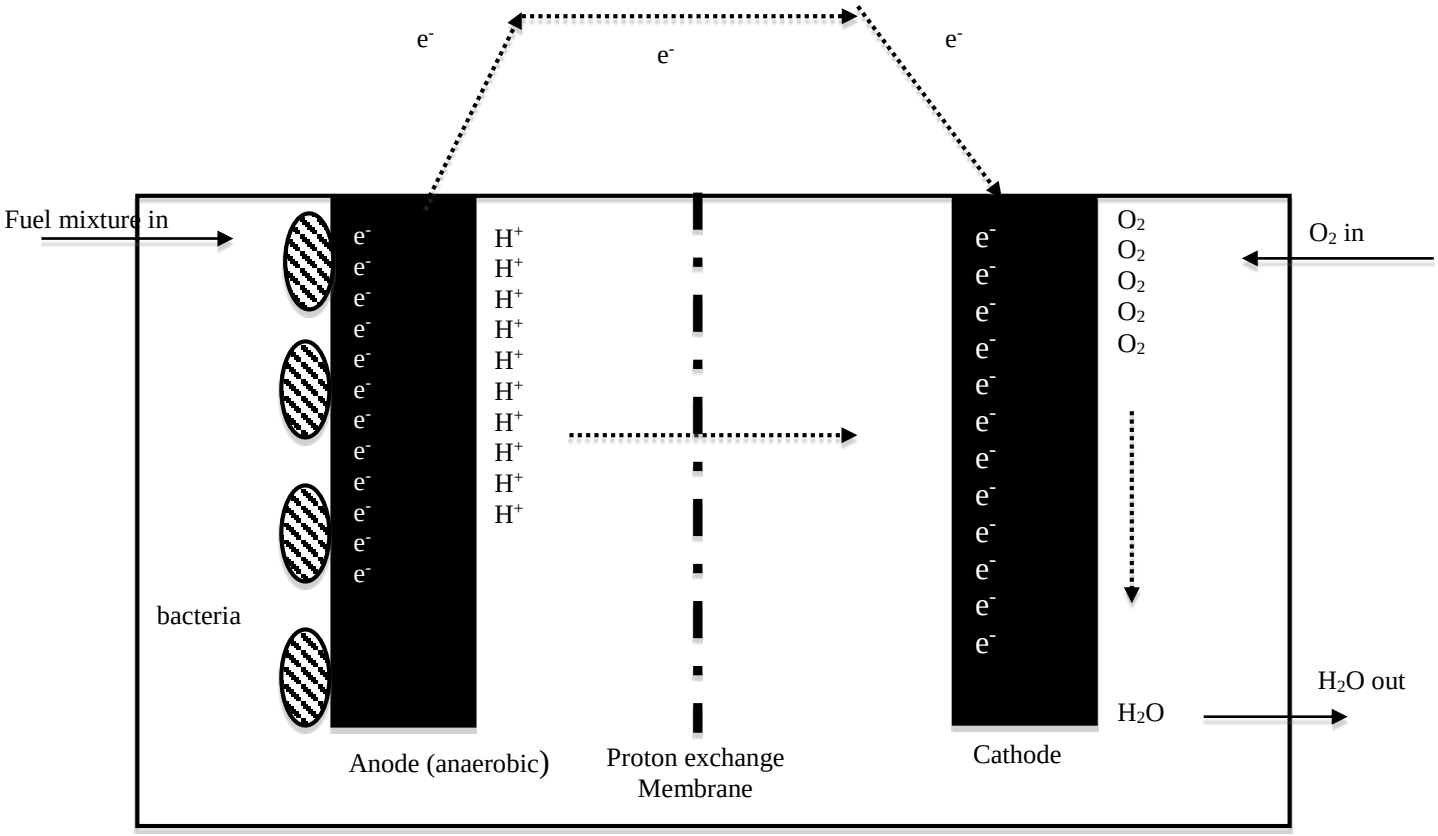


Figure 1.2. Figure 1.2 gives a schematic of a MFC and the reactions occurring within it. Bacteria growing on the anode or in the anode chamber oxidise the fuel entering the chamber to H^+, e^- and CO_2 (e.g. $C_2H_4O_2 + 2H_2O \rightarrow 2CO_2 + 8H^+ + 8e^-$). The e^- are transferred to the anode via extracellular electron transport pathways (the bacteria are using the anode as a terminal electron acceptor). The H^+ and e^- migrate to the cathode chamber where they reduce O_2 entering the chamber to H_2O ($2O_2 + 8H^+ + 8e^- \rightarrow 4H_2O$). This set of redox reactions generates an electrical current. On an electrochemical level MFCs are similar to a hydrogen fuel cell; the main difference is the bacteria in the anode chamber which allow them to use a wide variety of fuel sources (Rabaey and Verstreate 2005).

MFCs represent another method of harnessing the energy of biomass and have several different uses. The different uses of MFCs are discussed in section 1.3.

1.3 The uses of microbial fuel cells

The microbes colonising the anode mean that an MFC can use a wide range of compounds as a fuel source including proteins, sugars, organic acids, alcohols and various other complex molecules (Logan 2008). By extension of this versatility MFCS do not require a "pure" fuel source like the hydrogen fuel cells discussed in section 1.1, where impurities in the fuel source are likely to poison the catalyst (Lovely 2006). This suggests a role in bioremediation and this is an area where the potential role of MFCs has been investigated intensely, particularly in wastewater treatment as well as bioremediation of other contaminants, which are discussed in section 1.3.1 and 1.3.2 respectively.

1.3.1 Wastewater treatment by MFC

The traditional methods for removing organic material from wastewater are aerobic treatment, which consumes a considerable amount of energy (Rozendal *et al*., 2008) and anaerobic digestion, which produces biogas meaning it represents a source of renewable energy (Pham *et al*., 2006). MFCs represent a new method of harnessing the energy found in wastewater (Pham *et al*., 2006). The main advantage MFCs have is in the treatment of low chemical oxygen demand (COD), a measure of organic

material in wastewater based on how much oxygen is required to oxidise all the material to CO_2, expressed in mg O_2/L (Rozendal *et al*., 2008). A second advantage to using MFCs is that they can treat wastewaters at temperatures below 20 °C, which is where conventional anaerobic digestion begins to fail (Pham *et al*., 2006).

The ability of MFCs to electrogenically break down the organic matter in wastewater has been studied in considerable detail (Du *et al*., 2007). The purification of wastewater by MFC was first studied in 1991 where MFCs were used to purify sugar waste from water (Habermann and Pommer 1991). Since then the use of MFCs for the treatment of wastewater has been explored much more thoroughly.

MFCs have successfully been used to treat wastewater from the food processing industry (Oh and Logan 2005). In this study, high sugar wastewater from cereal processing was used to generate electricity with a two chambered MFC producing a power density of 81 ±7 mW/m^2 (the amount of power produced per m^2 of anode) during the first 200 h of operation and showed a 95% reduction in the COD of the wastewater after 400 h (Oh and Logan 2005). MFCs have been used to treat wastewater from a number of other diverse food processing industries; an MFC was used to treat wastewater from the chocolate industry where an MFC removed up to 75% of the dissolved COD and generated currents of up to 2.3 A/m^2 (Patili *et al*., 2009). MFCs have also been found capable of operating on a mixture of fatty acids; one study showed an MFC to produce a power density of 49 mW/m^3 (the power produced per litre of the total volume of the anode chamber) over a period of time (Freguia *et al*., 2010). Another study employed MFCs to treat brewery wastewater

(Feng *et al*., 2008). In this study 85-87% of the total COD (2,250± 418 mg/L) was removed depending on operating temperature (Feng *et al*., 2008) and power densities of 170 mW/m^2-205 mW/m^2 at 20°C to 30 °C respectively and was further increased to 438 mW/m^2 and 520 mW/m^2 when 50 mM and 200 mM respectively of phosphate buffer was added (Feng *et al*., 2008). This study therefore highlighted how variable factors such as temperature and pH can strongly affect the performance of an MFC, by adversely effecting bacterial growth highlighting one of the potential limitations of the technology i.e. factors adverse to bacterial growth can severely reduce the performance of a system. These studies show that MFCs remove a high amount of COD and therefore have a potential role in the purification of wastewater from a wide range of food processing industries.

MFCs can be used to treat domestic wastewater (Logan 2008). In 2004 Liu *et al* demonstrated that an MFC could both generate electricity and purify domestic wastewater (Liu *et al*., 2004). The fuel cell was found to remove up to 80% of the COD in the wastewater and generated power densities of up to 26 mW/m^2, with the amount of power generated depending on the COD of wastewater supplied, which ranged from 50-220 mg/L (Liu *et al.*, 2004). Other studies have shown similar results, for example a study in 2004 by Min and Logan used a flat plate MFC to remove 79% COD from domestic wastewater, while generating power densities of up to 43 mW/m^2 (Min and Logan 2004). In more recent studies the amount of power generated from domestic wastewater was increased by operating MFCs at mesophilic temperatures (30°C ±1) as opposed to ambient temperatures (23 °C ±1) where power

densities of up to 422 mW/m^2 were reported (Ahn and Logan 2010). This highlights how temperature is an important factor in the operation of MFCs.

In addition to treating wastewater from the food industry and domestic wastewater MFCs have been used to treat wastewater from other sources such as animal and agricultural wastewaters. An MFC was successfully used to treat swine wastewater (Min *et al*., 2005). The swine wastewater contained 8320 ± 180 mg/mL COD with 88% being removed by treatment with a MFC with power densities of 261 mW/m^2 (Min *et al.,* 2005) which shows that swine wastewater generated 79% more power than the domestic wastewater previously treated with the same system (Liu *et al*., 2004) due to the swine wastewater's higher COD (Min *et al*., 2005). A further example of the use of MFCs to treat agricultural wastewater was the use of a MFC to treat cow wastewater slurry (Yokoyama *et al*., 2006). Here an MFC removed 84% of the COD in slurry and generated a power density of up to 0.34 mW/m^2, which was low compared to other studies and was due to an inefficient MFC design (Yokoyama *et al*., 2006). The system used plain graphite anodes; these have a smaller surface area than other anode designs such as graphite brushes, which decreases bacterial load and reduces power output (Yokoyama *et al*., 2006). This highlights the importance of anode design to MFC performance.

The above studies all deal with the treatment of wastewater by MFC on a small, laboratory scale and show how MFCs are capable of removing organic components of wastewater while generating electricity at that scale. A number of pilot studies have been performed at larger scale, including the use of a MFC to treat brewery

wastewater at the Fosters brewery in Queensland Australia (www.microbialfuelcell.org 2012), the results of which are unreported, and at University of Connecticut where a MFC was built to treat wastewater on a large scale and found to remove 80% of the COD (Logan 2010). Other examples of MFCs used to treat wastewater on a larger scale include a multi electrode set up designed to treat domestic wastewater which generated power densities of up to 500 mW/m^2 from 20 L of domestic wastewater and removed 80% COD (Jiang *et al*., 2011). A 1000 L MFC was also developed and tested on winery wastewater (Cusick *et al*., 2011). This system produced a current of 7.4 A/m^2 and was found to remove 60% of COD from the wastewater (Cusick *et al*., 2011). More recently Zhang *et al*. demonstrated the usefulness of MFCs for treating wastewater on a larger, continuous scale. In this study two 4L tubular MFCs were used to treat wastewater in a municipal wastewater facility over a 400 day time period (Zhang *et al*., 2013). The fuel cells were found to remove 60-70% of the COD over a hydraulic retention time of 11 h which meant that they removed 0.4 kg/m^3/day of COD (Zhang *et al*., 2013).

It is therefore possible that MFCs will be used to treat wastewater on a commercial scale in the near future (Logan 2010, Rozendal *et al*., 2008, Zhang *et al*., 2013), although it seems MFCs are more useful as a means of purification than generation of power. The process of large scale wastewater treatment by MFC still requires further exploration.

1.3.2 The use of MFCs for bioremediation

In addition to purifying wastewater from a variety of sources (domestic, food processing, agricultural) MFCs have also been used in the bioremediation of a number of other substances.

A study by Luo *et al*. showed MFCs were capable of degrading phenol (Luo *et al*., 2008). Phenol is a common contaminant in effluents from a variety of industries including pesticide production, dye production and the pharmaceutical industry (Luo *et al*., 2008), therefore its removal is of interest to a variety of industries. In this study up to 95% of a 400 mg/L supply of phenol was degraded and electricity was generated at 9.1 mW/m^3 (Luo *et al*., 2008). This power density is low compared to that generated from MFCs used to treat wastewater and was due to the fact that phenol can inhibit the growth of bacteria at high concentrations even if those bacteria can use it as a substrate (Hill and Robinson 1975). It must be noted that the power produced by the system increased to 28.3 mW/m^3 when phenol was mixed with glucose, a more readily degradable substrate (Luo *et al*., 2008).

MFCs have also been used in the degradation of petroleum hydrocarbons contaminating groundwater (Morris *et al*., 2009). An MFC set up for this purpose removed 82% of the hydrocarbon contaminants (supplied at a concentration of 300 mg/L of diesel) over a 21 day time period and generated power densities of 31 mW/m^2 (Morris *et al*., 2009).

MFCs have also been shown to be capable of purifying leachate from landfills; leachate is heavily contaminated wastewater produced when water passes through decomposing waste (Greenman *et al*., 2009). During this study an MFC was found to produce power densities of 1. 38 mW/m^2 and removed COD at a rate of 48 mL/h (Greenman *et al*., 2009).

It has been found that MFCs can be used to remove uranium from contaminated groundwater (Gregory and Lovley 2005). In this study *Geobacter sulfurreducens* on the anode reduced the soluble U^{6+} to insoluble U^{4+} thereby precipitating the uranium from the contaminated ground water an allowing it to be easily removed by removing the electrodes from the system (Gregory and Lovley 2005). This method was able to recover up to 87% of the uranium removed from the groundwater by bacterial reduction (Gregory and Lovley 2005). Uranium is a common contaminant of groundwater that is beyond the scope of pump- and- remediation- based strategies (Riley *et al*., 1992) and supplying organic electron donors such as acetate to contaminated groundwater to promote reduction of U^{6+} by indigenous bacteria does not concentrate the reduced uranium which means it is difficult to remove. Therefore the use of MFCs represents a potential way to treat uranium- contaminated groundwater by precipitating uranium in an easy-to- remove form i.e. on the electrodes (Gregory and Lovley 2005).

The above studies show that MFCs are a viable method for bioremediation of a number of common pollutants and contaminants, because even though the yields of power are relatively low they remove a high percentage of the contaminants, which

is the primary concern with regards to bioremediation strategy. However it must again be noted that these experiments have all be conducted on a small, laboratory scale and MFCs as a bioremediation tool in large scale applications remain untested.

So far MFCs have been discussed in the context of removing biomass and generating electricity. The other method by which MFCs can harness waste biomass is by using them to produce biohydrogen.

1.3.3 The use of MFCs in the production of hydrogen from biomass

More than 90% H_2 is produced from fossil fuels. This is primarily performed by steam reforming; a process where steam reacts with hydrocarbons, most commonly methane, at temperature of >700 ºC and in the presence of a nickel catalyst to produce CO and H_2. The use of fossils results in the release of CO_2 contributing to climate change and are non-renewable (Logan 2009). It is therefore desirable to seek a renewable and green method of producing hydrogen gas. One such method is the electrolysis of water; this has high-energy requirements 5.6 kW/m^3 of H_2 costing $3 per kilo H_2 and has efficiencies ranging from 53%-75% (Ivy 2004). Therefore more efficient, less expensive sources of H_2 are required; one such source is biomass.

Fermentative techniques can be used to produce hydrogen from carbohydrates such as starch, cellulose and glucose (Nath and Das 2004) although there is an upper theoretical limit of 4 mol H_2/mol of glucose. In practice hydrogen is produced at

0.57- 2.2 mol H_2/ mol of glucose (Nath and Das 2004). MFCs represent a new method, non-fermentative method of producing hydrogen from biomass (Liu *et al*., 2005). Hydrogen production in an MFC was achieved by augmenting the electrochemical potential generated by the bacteria with an additional voltage of at least 250 mV, which leads to hydrogen being produced at the cathode and in this particular study acetate was used to produce hydrogen with 2.9 mol of hydrogen being generated per mol of acetate and could be improved upon with improvements in MFC design and further study (Liu *et al*., 2005). Studies discussed below give further examples of MFCs being used to produce H_2, where often the yield has been improved.

Call and Logan found that a high yield of H_2 could be achieved using a single chamber MFC (i.e. an MFC without a membrane, see section 1.4 for details, Call and Logan 2008). In this study a single chamber MFC was fed with 1 g/L of acetate and a potential of 0.8 V was applied to the system generating 3.12 ± 0.02 m^3 of H_2 per day. This was more than double the amount previously produced using 2 chambered MFC set ups (Call and Logan 2008). The higher yield of H_2 was due to the lowered internal resistances of the cell, due to the lack of an internal membrane (Call and Logan 2008). The system also produced purer H_2 due to the design exposing the anodic biofilm to O_2 between feeding cycles (Call and Logan 2008) thus limiting the action of methanogens (Call and Logan 2008), which have been found to be a common problem in this type of MFC setup (Tartakovsky *et al*., 2009). Other studies have also shown membraneless MFCs to be capable of producing high yields of H_2 when operating continuously (Tartakovsky *et al*., 2009). Here 3.9 mol of hydrogen

were produced for every mol of acetate fed into the cell (Tartakovsky *et al*., 2009). The finding that single chamber MFCs can be used for hydrogen production is important as it means less expensive setups can be used for the production of biohydrogen (Call and Logan 2008).

Recent developments in the technology have led to the production of higher H_2 yields, with yields approaching 100% being possible i.e. nearly 100% of the substrate fed into a fuel cell being converted into H_2 (Logan 2009). More recent studies have connected photosynthetic biohydrogen reactors to single chamber MFCs and, which yielded production of hydrogen 0.44 mmol/l/h, which was 15 * higher than that obtained from photosynthetic bio-reactors alone (Li *et al*., 2013).

H_2 production using MFCs offers several distinct advantages over the production of H_2 by fermentation. As can be seen from the above studies the yield of H_2 produced is typically much greater than that produced by fermentation. The non-fermentative nature of the process means that carbohydrate rich biomass is not needed (Liu *et al*., 2005) meaning that hydrogen can be produced by MFC from a wide range of waste biomass sources such as residential, agricultural and industrial wastewater (Logan 2008). In the studies above the carbon source used for hydrogen production was primarily acetate, which means MFCs can be coupled to fermentative process to utilise the fermentation end products to produce H_2 gas (Liu *et al*., 2005). The role of MFCs in wastewater treatment suggests that H_2 can be generated from the treatment of wastewaters with MFC (Liu *et al*., 2005).

The usefulness of MFCs for large scale hydrogen production is as yet unknown and requires further investigation. However it is thought that H_2 produced by MFC electrolysis could be sold off to offset the cost of wastewater treatment (Liu *et al*., 2005) and has more recently been found to be a cost effective method of hydrogen production with pilot scale studies having an effective cost of \$4.51 kg/H_2 which is less than the estimated merchant value of H_2 which is \$6 kg/H_2 (Cussick *et al*., 2010). Several pilot scale studies have been performed regarding the production of hydrogen by MFC. These studies include a twin tubular reactor on low COD domestic wastewater, which removed 80% COD and produced hydrogen at up to yields of 1.0 mol H_2/ mol COD (Gill-Carrea *et al*., 2013); another pilot scale study showed that a MFC treating winery wastewater yielded hydrogen at a maximum of 0.9 L H_2/L/day (Cusick *et al*., 2011). These successful pilot studies suggest that MFCs will see increased use for biohydrogen production on a larger scale in the future.

1.3.4 Use of MFCs to power remote devices

Another use for microbial fuel cells is to power remote devices found in locations off the national grid (Logan 2008) or where conventional batteries would prove an expensive logistical challenge to replace (Shantarem *et al.*, 2005).

Most studies where a MFC has been used to power a remote device use a specific type of MFC setup called a benthic unattended generator or BUG (Lovely 2006). BUGs are sediment MFCs that harness electricity from organic material in anaerobic,

aquatic sediments and can be potentially used for powering electronic devices at the bottom of the ocean and in other aquatic environments (Lovely 2006). Figure 1.3 illustrates the setup of a BUG.

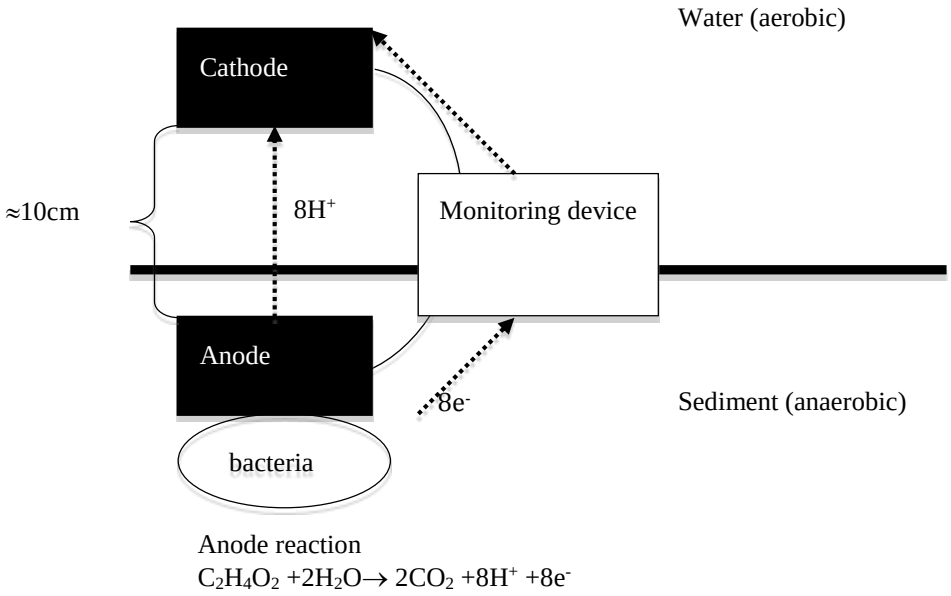


Figure 1.3 adapted from Lovley 2006. Figure 1.3 shows a schematic diagram of a BUG. The anode is inserted into the anaerobic sediment and the cathode is positioned approximately 10 cm above in the aerobic water. Bacteria colonise the anode and oxidise organic acids in the sediment to produce protons and electrons. These then travel to the cathode where they reduce oxygen to water and thus generate electricity. Equipment such as a sensor or monitor is connected to the BUG and powered by these redox reactions. The BUG has many potential applications in powering devices in aquatic environments.

Tender *et al*. first showed that BUG had a practical application by using the setup to produce power at levels greater than 0.025 W/m^2 in two different locations (Tender *et al*., 2002) and have since then been used for a number of different applications.

Shantaram *et al*. used BUG set ups for powering wireless sensor devices in aquatic environments (Shantaram *et al*., 2005). During this study energy generated by the MFCs was stored in a capacitor and used in short bursts when needed. The MFC set up effectively overcame the problems of using batteries to power these devices (Shantaram *et al*., 2005). Donovan *et al*. also showed that a BUG set up could be used in a fresh water environment to power a remote sensing device (Donovan *et al*., 2008). This shows that BUGs are usable in a variety of environments, as other studies have only shown BUGs being used in salt-water environments. A BUG device has been constructed that could sustain 36 mW per year which is the equivalent of 26 alkaline D cell batteries and was used to power meteorological buoys, allowing them to operate continuously without the need to replace batteries (Tender *et al*., 2008). Other examples of the use of MFCs for powering remote devices include the use of stacked submersible MFC to generate power densities of up to 294 mW/m^2 (Zhang and Angelidaki 2012) and a range of wireless sensors when coupled with a capacitor to store the power generated by the MFC (Dewan *et al*., 2010). The above examples show that MFCs are a useful device for powering remote devices and may see further use for this purpose in the future.

1.3.5 the advantages and disadvantages of MFCs

As shown by the above studies MFCS have been shown to be useful in a number of studies; however there are limitations to their use. The main advantages and

disadvantages to the use of MFC technology to harness the energy of biomass are discussed below.

The main advantages of MFCs are they can operate in areas of low COD and temperatures of less than 20 ºC which means they can be used to harness energy from biomass that cannot be harnessed by traditional anaerobic digestion (Pham *et al*., 2006). They have also been shown to remove high amounts of COD from wastewater and other sources on both a small and larger scale which suggests that MFCs are useful for wastewater treatment and bioremediation particularly in areas where traditional anaerobic digestion is not a viable option. Perhaps the most significant advantage of MFC technology is that they can efficiently produce a high yield of H_2 from a wide variety of substrates, which is thought to be one of the main uses MFCs will see in the future (Logan 2009). This has been shown to be a cost effective method of hydrogen production with costs being estimated at \$4.51 kg/H_2 produced by MFC which is less than the merchant value of H_2 which is \$6 kg/H_2 (Cussick *et al*., 2010). In terms of wastewater treatment MFCs are nearing the point where they could be employed practically and it has been predicted that an MFC could treat up to 144 L of wastewater/ day i.e. 0.5 g-COD/l/day (Lefebrve *et al*., 2011). The other main emerging use for MFCs seems to be to power remote, low power devices (Franks and Nevin 2010). In these situations they offer a distinct advantage as they negate the need for batteries, which is technically difficult and costly in remote devices (Franks and Nevin 2010).

The main disadvantage of MFCs is that they produce relatively low power densities of no more than several thousand mW/m^3 (Du *et al*., 2007, Logan 2009, Franks and Nevin 2010, Knight *et al*., 2013). This currently limits their usefulness to provide power and suggests that at present MFCs will be used to complement traditional wastewater treatment processes particularly in areas where temperature and COD is low rather than replacing them. However there has been a concerted effort to improve the system architecture of MFCs and thus increase their power output as discussed in section 1.4.

1.4 The design of MFCs; overcoming the limitations

A limiting factor of current MFCs is system architecture (Logan and Regan 2006). There have been many efforts to improve MFC design, which can be divided into three areas; anode design, cathode and catholyte design, and membrane design. The main goal however is to produce a system that maximizes power density and efficiency while being cost effective and easy to apply to large scale applications (Logan 2008).

1.4.1 Anode design

The important factors of an anode are that it is electrically conductive, inexpensive, has a high surface area and doesn't foul or decay within an MFC, therefore the most popular choice for anodic material is carbon (Logan 2008). Different forms of carbon have been used as MFC anodes to varying degrees of success. A particularly

successful anode design is the graphite fibre brush anode. These are graphite anodes with a brush like structure (see Figure 1.4) that have a very large surface area (Logan *et al*., 2007). Cells constructed with these anodes then inoculated with wastewater were found to produce power densities up to 1450 mW/m^2 compared to the 600 mW/m^2 power density generated by MFCs constructed with a plain carbon paper electrode (Logan *et al*., 2007). The higher power density produced is due to the increased surface area of the anode allowing for it to support a higher bacterial load. A typical graphite brush anode is shown in Figure 1.4.

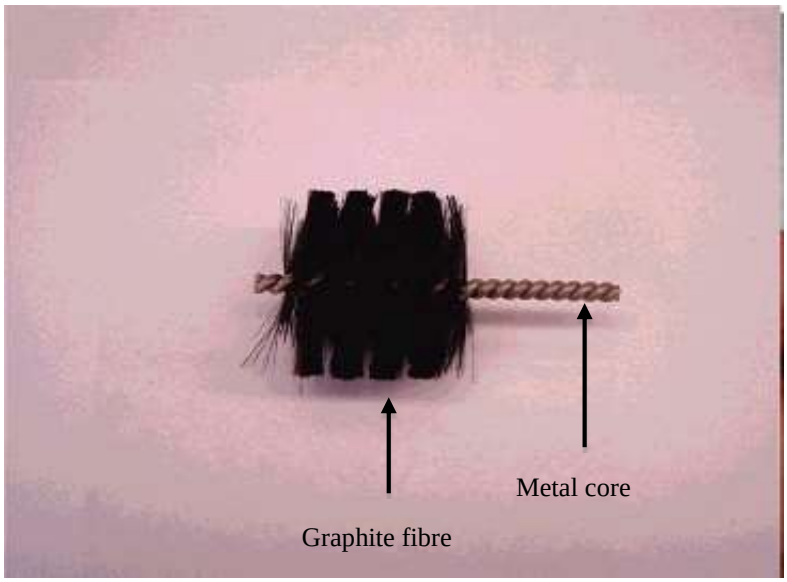


Figure 1.4 adapted from Logan *et al*., 2007 shows a graphite brush electrode. A graphite brush electrode consists of a number of graphite fibres wrapped around a non-corrosive metal core. This structure creates a very high surface area compared to a normal carbon paper electrode, therefore allowing for a higher bacterial load to develop on the anode, which allows the MFC to generate a higher power density.

Another useful anodic material is reticulated vitrified carbon (RVC), which has a low resistance, is highly porous (therefore has a high surface area) and is highly conductive (He *et al*., 2005). This has shown limited success, where a MFC built using this sort of anode produced power densities of 170 mW/m^2 , although the system was highly efficient in terms of wastewater treatment removing more than 90% of COD of the feedstock supplied (He *et al*., 2005).

Graphite granules have also been used as an anode in MFCs (Rabaey *et al*., 2005). Studies show that setups using graphite granules are capable of generating power densities of up to 90 mW/m^3 (Rabaey *et al*., 2005). Graphite granules have also been used to remove sulphite from a MFC setup (Rabaey *et al*., 2006). Here 514 mg/L/day of sulphite generating a power output of 101 mW/m^3 (Rabaey *et al*. 2006). Therefore graphite granules are clearly a useful anode; however they must be packed tightly in order to maintain good electrical conductivity and this, coupled with their irregular size means that only a small fraction of their surface area is available for bacteria to grow on (Logan 2008).

Based on the above studies it would seem that the graphite fiber brush electrode is one of the most effective designs as it is relatively cheap, has a high surface area, therefore produces high power densities and does not foul or corrode within the internal environment of an MFC. The graphite brush electrode has been used in a number of MFC systems including in combination with fiber glass separators to reduce the short circuiting of closely placed electrodes (Zhang *et al*., 2011).

The treatment of anodes with different substances in order to increase conductivity has been attempted to varying degrees of success. The most successful of these treatments has been treatment with ammonia; this increased the positive charge on the electrode that in turn resulted in an increase of power density from 1640 mW/m^2 to 1970 mW/m^2 (Logan 2008). More recent studies also include treatment of anodes with 4(*N*,*N*-dimethylamino)benzene diazonium tetrafluoroborate to increase nitrogen containing functional groups much like ammonia treatment (Saito *et al*., 2011) This produced power densities of 938 mW/ m2 27% higher than the untreated anode (Saito *et al*., 2011). Coating electrodes with a variety of metals and metal oxides has also been attempted, however this resulted in either an inhibition of electricity generation or a reduction in the amount of power generated (Logan 2008). This was possibly due to metal toxicity towards the bacteria.

In conclusion a large surface area is a critical factor with regards to anode design, with systems that have anodes with a larger surface area generating and sustaining higher power densities. Treatment of anodes has also been successful in increasing the power density produced by MFCs systems although the chemicals used to treat an anode must be selected carefully so as to avoid toxicity to the bacteria within the anode chamber.

1.4.2 Membrane design and membraneless MFCs

Membranes are used within an MFC to separate the liquid in the anodic and cathode chambers (Logan 2008). This serves as a barrier to dissolved oxygen, preventing it entering the anode chamber and therefore improves the columbic efficiency of the MFC (Logan 2008). They also prevent the flux of anodic material into the cathode chamber, which could cause fouling and subsequent inactivation of the cathodic catalyst (Logan 2008).

The most commonly used proton exchange membrane used in Fuel Cells is Nafion 117 (Logan 2008). Nafion is a tetrafluroethylenebased polymer developed in the late 1960s at the Dupont Corporation by modifying Teflon (www.permapure.com 2012). Another type of membrane used in several studies is a cation exchange membrane produced by Membrane International inc. (He *et al*. 2005, Rabaey *et al*., 2003) and is much thicker than a Nafion membrane (Logan 2008).

Bipolar membranes have also been used in MFCs to reasonable effect (Heijne *et al.* 2006); producing columbic efficiencies of 80-90% and energy recovery of 18-29% (Heijne *et al*. 2006). A bipolar membrane consists of anion and cation membranes joined in series and when a voltage develops water is split into H^+ and OH^- ions and balances the pH within the MFC (Logan 2008). Balancing pH within the MFC can increase the efficiency as various studies have shown acidification of MFC systems can result in a decrease in electricity production, due to its adverse effect on bacterial

growth (Franks *et al*., 2008).

However membranes are one of the most problematic aspects of MFC design as they are typically expensive when used in larger scale applications and have high resistance, which increases the internal resistance of the device and therefore decreases power output (Logan 2008). Unlike hydrogen fuel cells, MFCs do not require a membrane and therefore the concept of membraneless MFCs has been explored. An membraneless or single-chamber MFC is an MFC that lacks a partially permeable membrane (Logan 2008). A typical single chamber MFC design is illustrated in Figure 1.5.

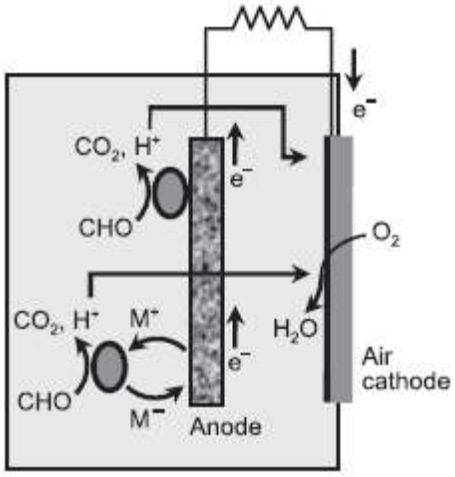


Figure 1.5 adapted from Watanabe 2008. It shows a typical schematic diagram of a single chamber MFC with an air cathode. The Cathode is placed with one side exposed to the air. There is no PEM between the anode and cathode. This will decrease the internal resistance of the system but may also decrease the columbic efficiency due to O_2 diffusion to the anode i.e. O_2 diffuses to the anode so the bacteria are either prevented from growing if they are strictly anaerobic or grow using the O_2 as opposed to the anode if they are factitively anaerobic or microaerobic. In either case less electricity is generated.

The general advantages of single chamber MFCs are that they lower the set-up cost of an MFC system (by removing the expensive membrane component) and lower the internal resistance of the system (Logan 2008). A single chamber MFC is also easier to apply to a large scale set up, due to its relatively simple architecture (Zhu *et al*., 2011). There have been several studies involving single chambered MFCs (Logan 2008). Jang *et al*. used a membrane-less MFC to enrich microbiological consortia for electrogenic bacteria (Jang *et al*., 2004). This system generated a stable current of 2 mA within a 4 week time period (Jang *et al*., 2004). Liu and Logan created a single chamber MFC system, which used glucose as an electron donor and the bacteria found in domestic wastewater as a biocatalyst (Liu and Logan 2004). In this system the removal of the membrane increased the power density from 262 to 491 mW/m^2 although it decreased the columbic efficiency i.e. the efficiency with which electrons are transferred within an electrical system from 55% to 12% due increased O_2 diffusion (Liu and Logan 2004). O_2 diffusion would decrease columbic efficiency as bacteria would transfer electron to the O_2 (using it as a TEA) rather than the anode. O_2 would also inhibit the growth of strict anaerobes in the anode chamber preventing them from transferring electrons to the anode. This study highlights the problem of O_2 diffusion resulting in a reduced efficiency that single chamber MFCs typically suffer from (Logan 2008). Zhu *et al*. recently constructed a single chamber MFC that generated power densities of 1641 mW/m^2 (Zhu *et al*., 2011). The very high power density was achieved by treating the anode with nitric acid (Zhu *et al*., 2011). Treatment with nitric acid worked in a similar fashion to the treatment of anodes with ammonia as discussed in section 1.4.1. This design was also scaled up and used to treat brewery wastewater due to its relatively simple and inexpensive architecture

(Zhu *et al*., 2011), although as previously stated the effectiveness of this setup has not be reported in the general scientific literature.

Therefore the main advantage of membrane-less MFC is that they decrease the internal resistance of the system, thereby increasing power density. They are also easy to scale up and have been used in a number of the pilot scale studies discussed in section 1.3. However there are also disadvantages to the use of a membrane less MFC. Single chamber MFC are prone to leakage of the anodic chamber, with the material diffusing into the cathode chamber, which can result in fouling and contamination of the cathode catalyst (Logan 2008). The lack of a membrane means that the anode chamber is also subject to oxygenation, which reduces columbic efficiency as discussed above. Therefore while single chamber MFCs usually produce more power than two chamber MFCs they are more difficult to work with.

1.4.3 Cathode design

There are a number of limitations at the MFC cathode which can severely affect and limit the performance of a system (Rismani *et al*., 2008). These limitations include activation losses (loss of energy due to high activation energy required for cathodic reaction, which is a problem with the air cathode setup), ohmic losses (loss of energy due to internal resistances of a system) and mass transport losses (which is loss of energy due to reactant depletion and/ or product accumulation, which slows reactions at the cathode) (Rismani *et al*., 2008). A number of different approaches have been taken in order to overcome these inherent difficulties.

Catalysts have been used to overcome the limitations of the air cathode (i.e. high activation energy) however typically this uses a precious metal catalyst, which is costly and therefore limiting to large scale applications of a MFC. In order to find a less expensive catalyst experiments with transition metal catalysts have been performed. A study by Zhou *et al*. used iron (II) phthalocyanine (FePc) and cobalt tetramethoxyphenylporphyrin (CoTMPP) based catalysts in order to investigate the effectiveness of transition metal catalysts for oxygen reduction (Zhou *et al*., 2005). A FePc- based cathode produced a maximum power output of 13.88 mW/m^3 and the CoTMPP- based cathode produced a maximum power output of 14.32 mW/m^3 (Zhou *et al*., 2005) This as comparable to the power produced by systems using a Pt. based catalyst (Zhou *et al*., 2005). It is therefore possible to run a MFC without using an expensive precious metal catalyst.

Another alternative to the problem of an air cathode using an expensive catalyst is to use an aqueous cathode as opposed to an air cathode. These have the distinct advantage of not requiring a catalyst (Logan 2008) and are based around solutions such as hexacyanoferrate ($Fe(CN)_6^{3-}$) or permanganate solutions (MnO_4^-) as the cathode electron acceptor (Logan 2008). Hexacyanoferrate has been effectively used as a catholyte solution in a number of studies, most notably in a tubular MFC system which produced power densities of 90 W/m^3 (Rabey *et al*., 2005). A 100 mM potassium permanganate solution produced a maximum power density of 115. 60 mW/m^2 compared to the 10. 20 mW/m^2 generated by an oxygen based cathode and the 25.62 mW/m^2 generated by a hexacyanoferrate based cathode (You *et al*., 2006).

These studies show that aqueous cathodes provide a viable alternative to air cathodes and in fact produce higher power densities. More recent studies have found potassium persulfate can also serve as a catholyte solution (Pandit *et al.*, 2011). It was used as a catholyte in a system which produced power densities of 101.7 mW/m^2 and was found to sustain the voltage for longer than permanganate; 40 h as opposed to 30 h (Pandit *et al*., 2011). It must be noted that the catholyte solution requires regular replenishment or regeneration, which means they are inconvenient for continuously operated systems. The catholyte solutions used in aqueous cathodes can also be toxic to the bacteria in the anode chamber although the architecture of the systems is such that there should theoretically be no contact between anode and cathode chambers, although leaks can occur.

A further approach to the problems and limitations associated with the cathode is the use of biocathodes. Biocathodes are where micro-organisms act as a biocatalyst for the cathodic reaction (Logan 2008). The effect of biocathodes was first discovered by Bergel *et al*. where a seawater biofilm formed in the cathode chamber of PEM fuel cell was found to catalyse the reduction of oxygen (Bergel *et al*., 2005). A number of studies have since been made into biocathode activity with regards to MFCs. Clauwaert *et al*., used a biocathode to produce a maximum power output of 65 W/m^3 in a system continuously fed with acetate at a rate of 1.5 kg $COD/m^{3.}$ (Clauwaert *et al*., 2007). The precipitation of Mn on the cathode was also observed overtime, which suggests a potential role for bioremediation using the biocathode (Clauwaert *et al*., 2007). Biocathodes have also been found to lower the internal

resistance of MFC systems; Chen *et al*. created a two chamber MFC set up with a biocathode and found that the internal resistance was reduced from 40.2 Ω to 14.0 Ω and the system generated power densities of 19.53 W/m^3 (Chen *et al*., 2008).

Biocathodes can also reduce nitrate and sulphate which suggests a further role in bioremediation as these components are a common component of many different wastewaters (He and Angenent 2006). Therefore biocathodes represent an inexpensive method of catalysing the cathodic reaction and can potentially increase the versatility of an MFC by allowing it bioremediate a wider range of substances. Biocathodes also allow for a greater range of substrates to be used as a catholyte and are low cost and represent a possible method to inexpensively scale up MFCs, although more experimentation and development is required (Huang *et al*., 2011).

These studies show that optimising the system architecture can significantly improve the performance of MFCs. However the most important factor of MFCs, which sets them apart from conventional fuel cells are the bacteria.

1.5 The microbiology of microbial fuel cells

Microbes capable of colonizing an MFC and producing electricity are termed electrogens or electrogenic bacteria. Electrogenic bacteria have been found across the bacterial kingdom and can be found in the five classes of proteobacteria,

firmicutes and acidobacteria (Logan 2009, Fedorovich *et al.*, 2009). Table 1.1 gives a summary of electrogenic bacteria discovered over the last decade.

Year	Bacteria	References
1999	*Shewanella putrefaciens* IR-I	Kim *et al*., 1999
2001	*Clostridium butyricum*	Park *et al*., 2001
2002	*Desulfuromonas acetoixidans*, *Geobacter maetallireducens*	Bond *et al*., 2002
2003	*Geobacter sulfurreducens*, *Rhodoferrax ferrireducens* A3 (*Aeromonas hydrophilia*)	Bond and Lovley 2003, Chaudhuri *et al*., 2003, Pham *et al*., 2003
2004	*Pseudomonas Aeruginosa* *Desulfobulbus propionicus* *Geopsychrobacter electrodiphilus*,	Rabaey *et al*., 2004 Holmes *et al*., 2004
2005	*Geothrix fermentans*	Bond and Lovley 2005
2006	*Escherischa coli* *Shewanella oneidensis* DSP10	Zhang *et al*., 2006 Ringeisen *et al*., 2006
2007	*Shewanella oneidensis* MR-1, *Pichia anomala*	Bretscherger *et al*., 2007 Prasad *et al*., 2007
2008	*Rhodopseudomonas palustris* DX-1, *Ochrobactum anthropi* YZ-1, *Desulfovibrio desulfuricans*, *Acidiphilum* sp. 3.2Sup5, *Klebsiella pneumoniae* L17, *Thermincola* sp. Strain JR,	Xing *et al*., 2008 Zuo *et al*., 2008 Borole *et al*., 2008 Zhang *et al*., 2008 Wrighton *et al*., 2008
2009	*Arcobacter butzleri* ED-1, *Arcobacter* L	Fedorovich *et al*., 2009
2012	Microalga *Dunaliella tertiolecta*, Microalga *Chlorella vulgaris*, *Citrobacter freundii*, *Vibrio azureus*, *Arcobacter nitrofigilis*, *Bacillus stratosphericus*,	Lankeniemi *et al*., 2012 Zhang *et al*., 2012

Table 1.1 adapted from Logan 2009 with species added from Fedorovich *et al*., 2009, Lankeniemi *et al*., 2012 and Zhang *et al*., 2012. Table 1.1 highlights the wide range of bacterial species showing electrogenic ability.

Table 1.1 shows that electrogenic ability is distributed widely throughout the bacterial kingdom and is found in highly unrelated species. It must be noted that a

high number of the electrogenic bacteria are also found to be capable of dissimilatory metal reduction. Dissimilatory metal reduction is where microorganisms use insoluble metals as terminal electron acceptors (Lovley 1993). The process is elaborated on in section 1.5.1.

1.5.1 Dissimilatory metal reduction

Dissimilatory metal reduction is a form of anaerobic respiration where a microorganism oxidizes an organic substance, typically an organic acid or short chain alcohol and reduces a solid insoluble terminal electron acceptor such as Fe^{3+} or Mn^{4+} via extracellular electron transfer (Lovely 1993). Like electrogenesis, dissimilatory metal reduction is found throughout the bacterial kingdom and archeal kingdom as well (Weber *et al*., 2006). Fe^{3+} was thought to be one of the first extracellular electron acceptors used by bacteria (Varges *et al*., 1998) with the organisms most closely related to the last common ancestor (*Pyrobaculum islandicum* and *Thermotoga maritime*) shown to be capable of utilizing Fe^{3+} as a terminal electron acceptor (Varges *et al*., 1998). On modern earth dissimilatory metal reduction is an important part of biogeochemical cycling both of transition metal elements and of carbon in anaerobic and microaerobic environments where bacteria couple the oxidation of a carbon source with the reduction of an insoluble metal ion such as Fe^{3+}(Weber *et al*., 2006). Figure 1.6 illustrates the principle.

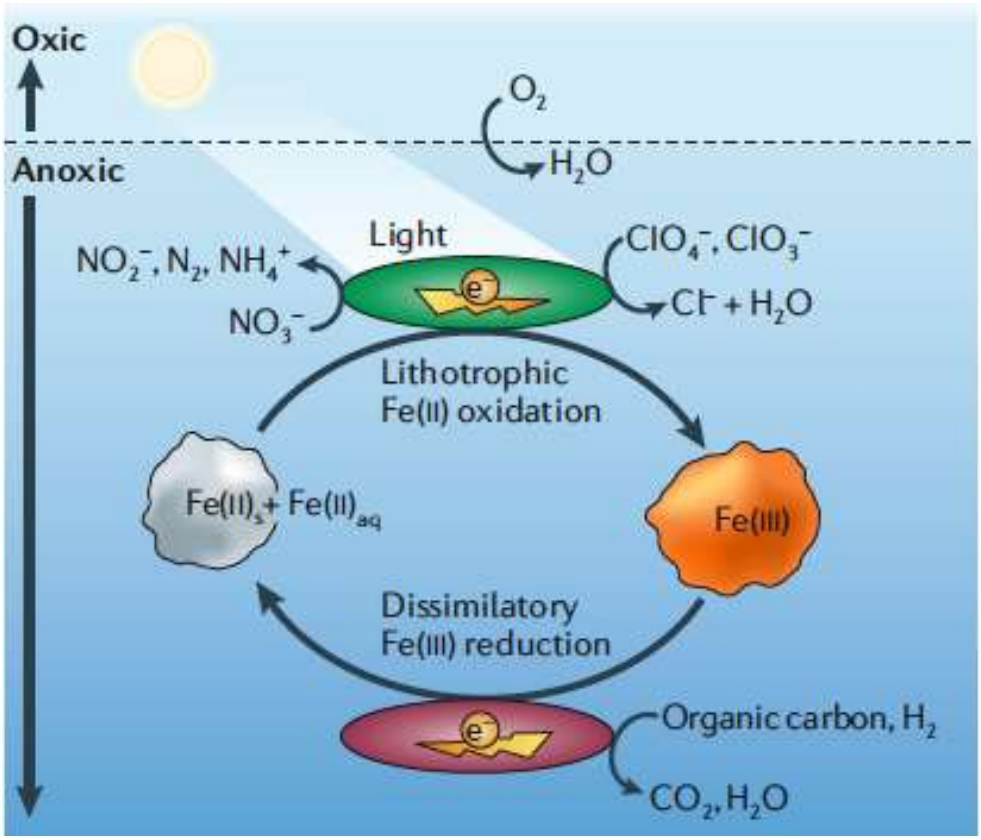


Figure 1.6 adapted from Weber *et al*., 2006. The figure shows how lithotrophic bacteria oxidise Fe^{2+} to Fe^{3+} and couple this to the reduction of NO_3^- to NO_2^-. Heterotrophic dissimilatory metal reducers then couple the reduction of Fe^{3+} to Fe^{2+} with the oxidation of complex organic carbon to CO_2 and H_2O. Therefore dissimilatory metal reduction is important in cycling both carbon and metal ions in anaerobic and microaerobic environments.

While iron is used in the examples above it is by no means the only metal cycled by dissimilatory metal reduction. Microorganisms that reduce Mn^{4+}, U^{6+}, Se^{6+}, Cr^{6+} have all been found (Lovley 1993) where they play a similar role in cycling these elements in anaerobic environments. Bacteria that reduce U^{6+} are also of interest from a bioremediation point of view and they have been found to reduce uranium levels in contaminated water by 90% (Finneran *et al*., 2002).

There are many different dissimilatory metal reducers; two of the best characterised are *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1, which are of particular interest as they have been both been studied as electrogens, with *Geobacter sulfurreducens* in particular serving as something as a model organism for electrogenesis.

Geobacter sulfurreducens is a Gram negative, rod shaped bacterium and member of the δ proteobacteria family. It was isolated from the anaerobic sediment of a hydrocarbon-contaminated ditch in Norway (Caccavo Jr. *et al.*, 1994). It is an obligate anaerobe and capable of utilising both Fe^{3+}, sulfur, fumarate and malate as sole terminal electron acceptors (Caccavo Jr. *et al*., 1994). The bacterium can utilise acetate and hydrogen as an electron donor (Caccavo Jr *et al*., 1994). The bacterium has since been found to be an electrogen (Bond and Lovley 2003) when it was first demonstrated to generate currents of 65 mA/m^2 when grown in pure culture, which at the time of the study was greater than that reported for previous MFC systems (Bond and Lovley 2003). The bacterium was found to produce nanowires (Regurea *et al*., 2005) and genome sequence analysis showed it to contain 111 outer membrane c type cytochromes (Methe *et al*., 2003). Two of these are of importance in extracellular electron transfer by the bacteria (the key physiological process behind both dissimilatory metal reduction and electrogenesis) and will be discussed in more detail in section 1.6

Shewanella oneidensis MR-1 are Gram negative, rod shaped bacteria motile by means of polar flagella (Venkasteswaren *et al*., 1999). They are members of the family γ proteobacteria (Venkasteswaren *et al*., 1999). Like *Geobacter sulfurreducens* they were found to be electrogenic in pure culture (Kim *et al*., 2002) when tested in am MFC system. The bacterium has also been found to produce nanowires (Gorby *et al*., 2006) and the genome sequence revealed a high number (42) of outer membrane cytochromes (Heidelberg *et al*., 2002).

The high number of outer membrane c type cytochromes and nanowires present in both *Geobacter* and *Shewanella* suggest that these proteins are an important part of extracellular electron transfer.

1.6 Extracellular electron transfer

Extracellular electron transfer is where bacteria transfer electrons across the outer cell membrane to an insoluble terminal electron acceptor. The process is important in both dissimilatory metal reduction and electrogenesis, where insoluble metal ions and an anode serve as the terminal electron acceptor respectively. The three methods of extracellular electron transfer are:

1. Direct contact; Outer membrane c type cytochromes transfer electrons across the outer membrane to an insoluble electron acceptor
2. Formation of a conductive biofilm; bacteria produce nanowires and secrete c-type cytochromes to form an electrically conductive extracellular matrix that facilitates the transfer of electrons across a biofilm to the insoluble electron acceptor. It must be noted that this is not a distinct method of extracellular electron transfer but rather an extension of the direct contact method.
3. The production of extracellular electron shuttles; the bacteria produce soluble compounds with a variable redox state that it uses to shuttle electrons across the outer cell membrane.

The three different methods of extracellular electron transfer are discussed in detail in section 1.6.1-1.6.3.

1.6.1 Direct contact

Direct contact is where the bacteria must be in direct contact with an electron acceptor in order to utilise it during anaerobic respiration. Many bacteria are capable of the process although it has been most extensively studied in *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1. C type cytochromes have been found to be of great importance during this process.

C type cytochromes are found in nearly all organisims where they are involved in electron transport and other redox processes (Shi *et al*., 2007). The proteins have at least 1 c type haem group joined by covalent linkage to the amino acid side chains and co-coordinated to cysteine molecules by thioester bonds (Shi *et al*., 2007). C type cytochromes play a role in the electron transport chain, where the variable redox state of the haem group allows for electron transfer between either different proteins or to a TEA. As previously mentioned genome sequencing has recently shown c type cytochromes to be present in high numbers in *Geobacter sulfurreducens* (Methe *et al*., 2002), *Shewanella oneidensis* MR- 1 (Heidelberg *et al*., 2002) as well as in other metal reducing bacteria such as *Desulfovibrio vulgaris* (Heidelberg *et al*., 2004), which suggests the importance of c type cytochromes in extracellular electron transfer. C type cytochromes and their role in extracellular electron transfer in *Geobacter sulfurreducens* and *Shewanella oneidensis* will be discussed in more detail below.

1.6.1.1 *Geobacter sulfurreducens*

A large number of c type cytochromes have been found in *Geobacter sulfurreducens* and a number of them are thought to play a role in extracellular electron transfer. A 300 kDa NADH dependent Fe^{3+} reductase has been found to be associated with the outer membrane *of Geobacter* (Magnuson *et al*., 2000). The reductase consists of 5 major proteins including a 89 kDa C-type cytochromes which was shown to transfer electrons to Fe^{3+} (Magnuson *et al*., 2001). This protein is thought to be unique to

Geobacter displaying only 20% homology with c type cytochromes in other bacteria (Magnuson *et al*., 2001).

Other c type cytochromes with a role in extracellular electron transfer have been identified in *Geobacter sulfurreducens*. The proteins OmcB and OmcC are expressed in *Geobacter* grown under anaerobic conditions with Fe^{3+} serving as the electron acceptor (Leang *et al*., 2003). OmcB was found to be more twofold more abundant; OmcC and OmcB deletion mutants were found to lack the ability to reduce Fe^{3+} whereas OmcC deletion mutants showed an ability to reduce Fe^{3+} comparable to that of the wild type. (Leang *et al*., 2003). Further studies have shown that OmcS, a 50 kDa hexahaem protein and OmcE, a 30 kDa protein are both essential for Fe^{3+} reduction as mutants lacking either of these proteins were unable to reduce Fe^{3+} (Mehta *et al*., 2005). OmcZ exists as a large (50 kDa) subunit and small (30 kDa) subunit (Inoue *et al*., 2011) and has been found to be important in current generation in MFCs (Nevin *et al*., 2009) with OmcZ deletion mutants being incapable of producing current (Nevin *et al*., 2009). It must be noted that OmcS and OmcE are down regulated in *Geobacter sulfurreducens* harvested from an MFC (Nevin *et al*., 2009), which suggests that while similar, the electron transfer pathways for dissimilatory metal reduction and electrogenesis are not identical.

1.6.1.2 *Shewanella oneidensis* MR-1

Myers and Myers first demonstrated the importance of c type cytochromes in metal reduction by *Shewanella oneidensis* MR-1 when a study showed 80% of membrane bound c type cytochromes were localised to the outer membrane under anaerobic conditions where Fe^{3+} served as a terminal electron acceptor (Myers and Myers 1992). Several distinct cytochromes have been isolated in *Shewanella oneidensis* MR-1. OmcA is an 83 kDa decahaem outer membrane c type cytochrome encoded for by the gene *omcA* (Myers and Myers 1998); other decahaem c type cytochromes include MtrA, MtrB and MtrC (Pits *et al*., 2003, Belieav *et al*., 2001). MtrA is a periplasmic protein that is part of the electron transport pathway for Fe^{3+}/ Mn^{4+} reduction whereas as MtrC is an outer membrane protein that forms part of a terminal Fe^{3+} reductase or at least required for its assembly as strains lacking MtrC show decreased Fe^{3+} reductase activity (Belieav *et al*., 2001). Further studies have shown that MtrC mutants as well as MtrA and MtrB mutants display a decreased ability to reduce insoluble electron acceptors and generate current when compared to wild type *Shewanella oneidensis* (Bretschger *et al*., 2007). In this situation mutants displayed nominal current density compared to that of 15 mA/cm^2 displayed by wild type *Shewanella*, although the mutants did generate comparable levels of current when complemented (Bretschger *et al*., 2007), therefore showing that these proteins are important for the reduction of insoluble terminal electron acceptors as is OmcA (Gorby *et al*., 2006, Coursolle and Gralnick 2010). OmcA deletion mutants were found to be incapable of reducing Fe^{3+} and generating current in an MFC (Gorby *et al*., 2006). It is clear that these proteins do not exist in isolation and instead interact

and complex together in a pathway dedicated to facilitating extracellular electron transfer. One possible arrangement of the proteins is illustrated in Figure 1.7.

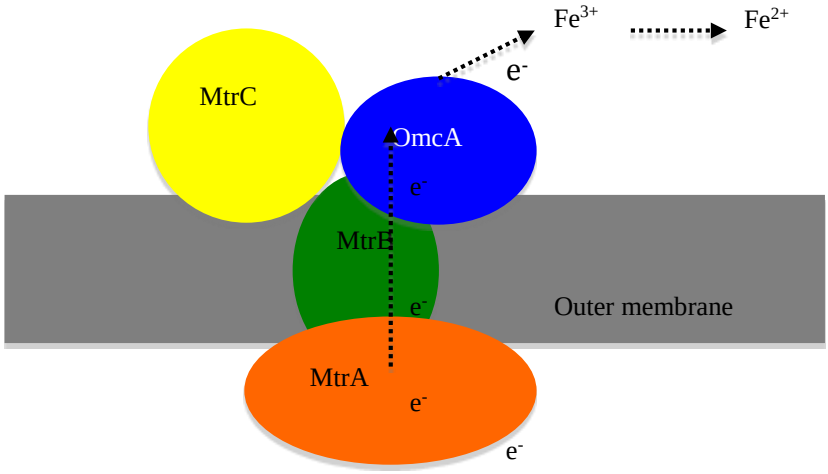


Figure 1. 7 adapted from Shi *et al*., 2007. Figure 1.7 shows a schematic diagram depicting the possibly arrangement of *Shewanella oneidensis* c type cytochromes that are involved in extracellular electron transport. In this model the periplasmic protein MtrA transports electrons to MtrB, which transports the electrons across the outer membrane to the MtrC/OmcA complex and then transports the electrons to an insoluble electron acceptor such as Fe^{3+}.Deletion mutant studies have shown that MtrC and OmcA are essential to this process with bacteria lacking these proteins being significantly impaired in their ability to reduce Fe^{3+} and generate current within a MFC.

The above examples show that c type cytochromes are an essential part of the direct contact method of extracellular electron transfer. They do not explain how these bacteria are able to form biofilms up to 50µm thick (Franks *et al*., 2008) as the pathways described above would only support transfer of electrons directly from cell

to electron acceptor not throughout a biofilm. Therefore in order to facilitate the transfer of electrons over long distances these bacteria produce electrically conductive appendages known as nanowires, which help them to form biofilms with an electrically conductive extracellular matrix.

1.6.2 Nanowires and the formation of an electrically conductive extracellular matrix

Nanowires are electrically conductive pili capable of transferring electrons over long distances (Reguera *et al.*, 2005). Nanowires are produced by bacteria under low oxygen tension, typically less than 2% (v/v) O_2 and are important in transferring electrons to insoluble electron acceptors (Regurea *et al*., 2005, Gorby *et al*., 2006). Recent studies of the nanowires of *Geobacter sulfurreducens* suggest that the mechanism of conduction is similar to that observed in metallic wires, which is in direct contrasts to previously observed mechanisms of biological electron transfer i.e. electron tunnelling and transfer between closely associated molecules (Malvankar *et al*., 2012). This metallic like conductivity (i.e. through delocalised electrons) is thought to be due aromatic amino acids (which have a cloud of partially delocalised electrons surrounding the aromatic side chain) with mutagenesis studies showing the substation of aromatic amino acids for alanine significantly reducing nanowire conductivity (Malvankar *et al*., 2012). While nanowires were first characterised in dissimilatory metal reducing bacteria, many different types of bacteria have been found to produce nanowires. Genes for nanowires have been found in photosynthetic cyanobacteria and heterotrophic sulphate reducing bacteria (Gorby *et al*., 2006) and

more recently in the bacteria *Acidithiobacillus ferrooxidans* (Li and Li 2013). In these systems nanowires are thought to act as a means as distributing excess electrons to metabolites where no immediate terminal electron acceptors are available (Gorby *et al*., 2006) and therefore are likely to play a broader role in anaerobic respiration and CO_2 fixation in anaerobic communities of bacteria (Gorby *et al*., 2006). Due to the nanowires of *Geobacter* and *Shewanella* being well characterised as is their role in electrogenesis and dissimilatory metal reduction a discussion on nanowires in these bacteria follows.

The *pil*A gene of *Geobacter sulfurreducens* encodes for monolateral pili that were found to be essential for the reduction of insoluble Fe^{3+} (Regurea *et al*., 2005) as they helped establish contact with the insoluble ions and facilitate the transfer of electrons to them. Figure 1.8 shows *Geobacter sulfurreducens* growing on insoluble Fe^{3+}.

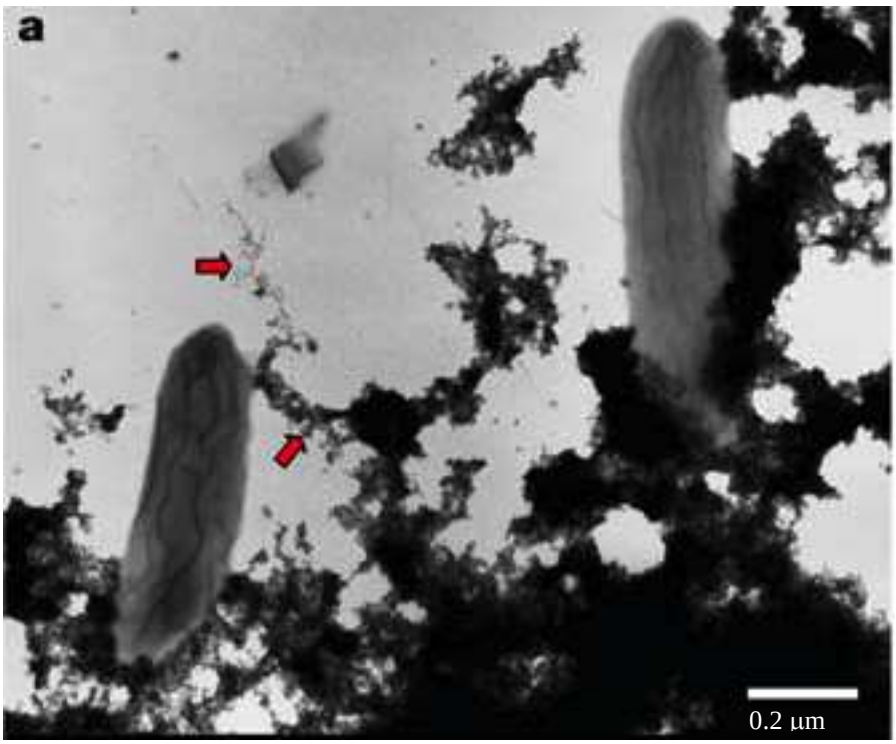


Figure 1.8 adapted from Regurea *et al*., 2005. Figure 1.8 shows a transmission electron micrograph (scale bar 0.2 μm). The arrows indicate pili and the images clearly shows poorly crystalline Fe^{3+} oxide accumulated along the pili. The nanowries are associated with c type cytochromes and facilitate the transport of e- from c type cytochrome to distant source of Fe^{3+}, therefore allowing the bacteria to fully utilise Fe^{3+} as a terminal electron acceptor..

It was found that while the pili were essential for insoluble Fe^{3+} reduction they were not required for the reduction of soluble electron acceptors such as fumarate or Fe^{3+} partially solubilised by the use of a chelating agent (Regurea *et al*., 2005). Further studies have shown that the nanowires of *Geobacter sulfurreducens* allow for the development of thick (up to 50 μm) biofilms on the anode (Regurea *et al*., 2006) and are up regulated in current generating cells harvested from the anode (Nevin *et al*., 2009). This explains how MFCs seeded with *Geobacter sulfurreducens* produce such high current as the nanowires allow large numbers of *Geobacter* to grow on and transfer electrodes to the anode, even over a long distance (Regurea *et al*., 2006,

Nevin *et al*. 2009). This information coupled with the information about c type cytochromes allowed for the development of this model of a *Geobacter sulfurreducens* anodic biofilm.

1.6.2 The anodic biofilm of *Geobacter sulfurreducens*

Geobacter sulfurreducens have been found to form thick (50 μm) biofilms on the anode (Franks *et al*., 2008). Transcriptomic analysis of anode associated *Geobacter* have shown an increase in OmcB, OmcZ and PilA within an anodic biofilm (Nevin *et al*., 2009) which suggests that these two c type cytochromes and nanowires are important in transferring electrons from the bacteria to the anode (Regurea *et al*., 2006, Nevin *et al*. 2009); deletion of OmcZ and PilA inhibited the bacteria's ability to produce current (Nevin *et al*., 2009). Further studies show that OmcZ is distributed throughout the biofilm (Richter *et al*., 2009) and along with the nanowires facilitates transfer of electrons across the biofilm, whereas OmcB was found to mediate electron transfer at the biofilm- anode interface (Richter *et al*., 2009).

Therefore it is proposed that *Geobacter sulfurreducens* forms a thick anodic biofilm capable of generating current due to nanowires and OmcZ forming a conductive extracellular matrix that transfers electrons from distance cells towards the anode where OmcB mediates transfer between the biofilm and the anode, therefore allowing all cells of the biofilm to use the anode as terminal electron acceptor and therefore generate current (Nevin *et al*., 2009, Richter *et al*., 2009). An example of a *Geobacter sulfurreducens* anodic biofilm is shown in Figure 1.9.

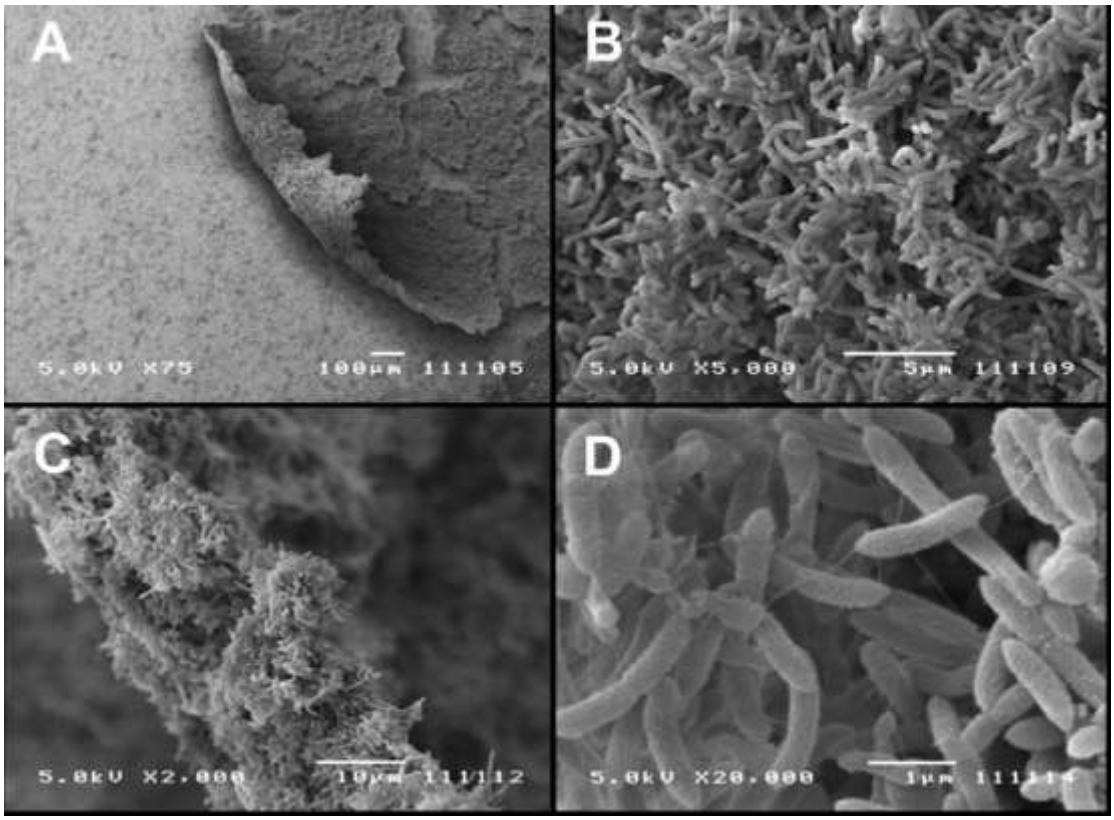


Figure 1.9 adapted from Richter *et al*., 2008. The figure shows scanning electron micrographs of Geobacter *sulfurreducens* anodic biofilm at different magnifications. Images B) and C) show a thick, highly differentiated biofilm that is typical for mature, current producing *Geobacter sulfurreducens* anodic communities. The thick biofilm is supported by the conductive extracellular matrix created by nanowires and OmcZ. Image D) shows the individual nanowires connecting the cells together. These nanowires transport electrons throughout the biofilm and allow all bacteria to utilise the anode as terminal electron acceptor with equal effectiveness.

Shewanella oneidensis MR-1 also produces nanowires, which are most likely the product of the *pil*A or *gsp*G gene of the type II secretion pathway (Gorby *et al*., 2006). Like the nanowires of *Geobacter*, the nanowires of *Shewanella* facilitate the

transfer of electrons to insoluble metal ions. The morphology of *Shewanella* nanowires is shown in the following images.

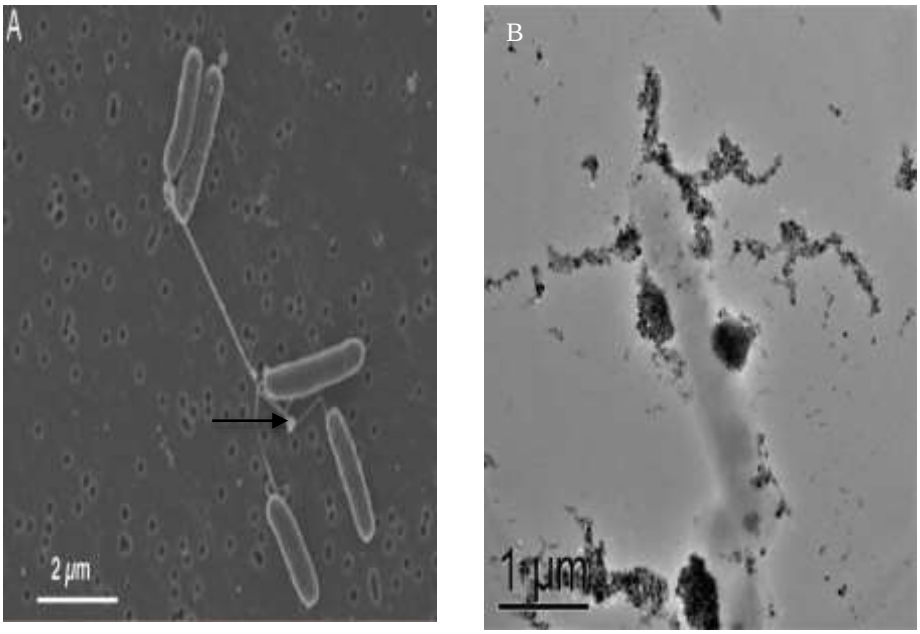


Figure 1. 10 adapted from Gorby *et al*., 2006. Image A) a scanning electron micrograph of *Shewanella oenidensis* nanowires. These nanowires are thicker and straighter than those of *Geobacter* and seem to form node like structure (shown by arrows). Image B) *Shewanella* with particules of insoluble Fe^{3+} aggregated along the nanowires. The nanowires work with C type cytochromes MtrC and OmcA to transport electrons across the outer membrane and facilitate contact with distant metal ions, therefore allowing the bacteria to effectively utilise these ions as a terminal electron acceptor.

The nanowires of *Shewanella oneidensis* are thought to interact with the cytochromes MtrC and OmcA (Gorby *et al*., 2006) as mutants lacking these proteins were found to produce nanowires that were poorly conductive (Gorby *et al*., 2006). The exact interactions between these proteins are unknown, however one possible

method is they interact with the MtrC-OmcA complex as seen in Figure 1.7 and help facilitate transfer of electrons from that complex over a long distance.

How these components interact to allow *Shewanella oenidensis* to form an anodic biofilm is as yet unknown; however imaging of the *Shewanella* biofilm shows a thick biofilm with nanowires connecting the cells together and to the anode. Therefore it is plausible that *Shewanella oneidensis* form a conductive extracellular matrix that facilitates the transfers of cells long distances across the biofilm to the anode. This is likely when it is considered that both bacteria contain similar outer membrane c type cytochromes and both produce nanowires, although further study would be required to verify this. An anodic biofilm of *Shewanella oneidensis* is shown in Figure 1.11.

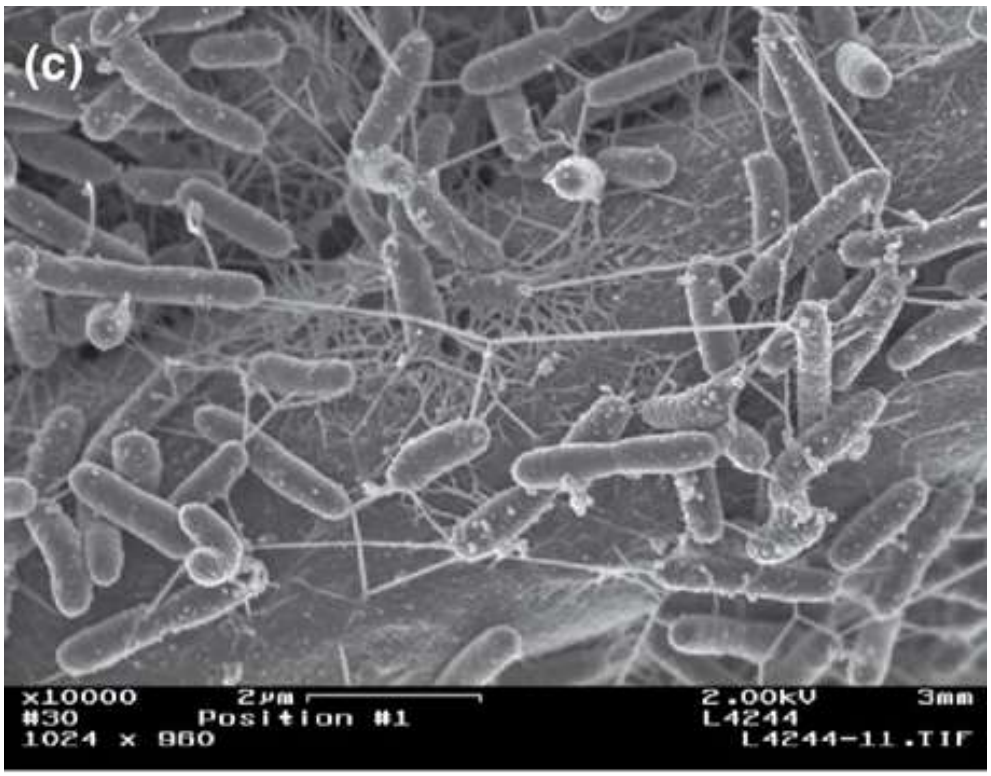


Figure 1.11 adapted from Logan and Regan 2006. The image shows a scanning electron micrograph of *Shewanella oneidensis* growing on an anode linked together by nanowires. This suggests that the bacterium forms a similar conductive extracellular matrix to *Geobacter sulfurreducens*, although further study is required to verify this.

While *Shewanella oneidensis* is thought to form a conductive biofilm, MFCs seeded with a pure culture of *Shewanella* have shown a high amount of planktonic phase growth (Lanthier *et al*., 2008). This suggests that the bacteria may employ multiple strategies for the transfer of electrons to the anode (Lanthier *et al*., 2008) such as the secretion of flavins (Marsili *et al*., 2008).

The direct contact/ conductive biofilm is perhaps the most well characterised method of extracellular electron transfer by bacteria in a MFC due to these cultures typically producing high current. They are not the only method of extracellular electron transfer, small soluble compounds known as electron shuttles are also a method of extracellular electron transfer.

1.6.3 Electron shuttles as a means of extracellular transportation

The conductive biofilm and direct contact mechanisms of electrogenesis are not the only mechanisms of electrogenesis; the other main mechanism of electrogenesis is the use of extracellular electron shuttles or mediators. Mediators are small, soluble compounds with a variable oxidation states that can carry electrons from a cell to an insoluble electron acceptor (Logan 2008). Several different bacteria have been found to perform electrogenesis using mediators; these are shown in Table 1.2.

Bacteria	Mediators
Eschericha coli	Hydrogenase (with neutral red added)
Pseudomonas aeruginosa	Pyocyanin, phenazine
Shewanella putrefaciens	Quinones
Erwinia dissolvens	Unknown
Shewanella oneidensis	Flavin mononucleotide riboflavin

Table 1.2 adapted from Rabaey and Verstraete 2005 and Velasquez-Or *et al,* 2010. A variety of different microorganisms are shown to employ a variety of mediators. *E. coli* is not noted as an electrogen, however it can be made electrogenic by the addition of additional mediator compounds and recent studies have found it can adapt to a MFC environment becoming electrogenic without an additional supply of mediator (Zhang *et al*., 2006).

The most extensively studied mediator producing bacteria are *Pseudomonas aeruginosa*, which produces pyocyanin/ phenazine and *Shewanella oneidensis*, which produced flavins.

Pseudomonas aeruginosa was found as the dominant species in a MFC that maintained a power density of 4.31 W m^{-2} (Rabaey *et al*., 2004). *Pseudomonas aeruginosa* cells transfer electrons to the anode by pyocyanin and phenazine-1-carboxamide (Rabaey *et al*., 2005). Mutant strains unable to produce these compounds generated only 5% the power of wild type strains (Rabaey *et al*., 2005) although upon addition of exogenous pyocyanin and phenazine power output was restored to 50% that of wild type levels and was found to improve electron transfer by other bacterial species (Rabaey *et al*., 2005). This suggests a potential

biotechnological use for pyocyanin and phenazine harvested from *P. aeruginosa* in boosting the power output of MFC systems (Rabaey *et al*., 2005).

Shewanella oneidensis MR-1 have been found to produce flavins (Marsili *et al*., 2008). Flavins are a group of organic compounds based around a petridine compound (Berg *et al*., 2012). Flavins are capable of alternating between oxidised and reduced forms states, which means they are well suited to serve as electron shuttles in MFCs (Berg *et al*., 2012). In this study riboflavin and riboflavin 5' phosphate were isolated from the biofilm supernatant (Marsili *et al*., 2008). Removal of these mediators from the biofilm reduced the rate of electron transfer to the anode by 70% (Marsili *et al*., 2008). The production of flavins by *Shewanella* is curious because, as discussed in section 1.6.1 and 1.6.*2 Shewanella oneidensis* are thought to engage in extracellular electron transfer by direct contact mechanisms, therefore there should be no need for mediators. However recent studies have shown that the two methods of extracellular electron transfer may co-exist. Recent studies have shown that the outer membrane cytochromes MtrC and OmcA are essential for the reduction of these flavins (Coursolle *et al.*, 2010), with mutant strains being unable to grow into current producing biofilms (Coursolle *et al*., 2010). This suggests that flavins in *Shewanella* may transport electrons throughout the anodic biofilm, which suggests that the conductive extracellular matrix of *Shewanella* anodic biofilms is quiet distinct from that of *Geobacter sulfurreducens* discussed in section 1.6.2. The production of flavins would also explain how *Shewanella oneidensis* could proliferate in high numbers in the planktonic phase of the anodic chamber of the MFC (Lanthier *et al*., 2010). The flavins could transfer electrons from bacteria in the

liquid phase to the anode, therefore allowing the bacteria to still use the anode as an electron acceptor despite not being attached to it.

The above examples show how extracellular electron transfer is not the result of one homogenous process but rather can be the result of different processes and that these processes are not as distinct as originally thought, i.e. cytochromes and extracellular electron shuttles can work together to maintain an anodic biofilm. There are also likely to be novel methods of extracellular electron transfer. Two such bacteria, thought to process novel methods of extracellular electron transfer are the bacteria *Arcobacter butzleri* ED-1 and *Arcobacter* L, which were found as the dominant species in acetate fed MFC (Fedorovitch *et al*., 2009). The genus *Arcobacter* and the *Arcobacter* isolated from the MFC are detailed in section 1.7 onwards.

1.7 The isolation of *A. butzleri* ED-1 and *Arcobacter* L from an acetate fed MFC

Arcobacter butzleri ED-1 and *Arcobacter* L were the first ε proteobacteria found to be capable of electrogenesis (Fedorovich *et al*., 2009). Previously *Arcobacter spp.* had been isolated from the anode of a formate fed MFC, although in this study the *Arcobacter spp*. were found to consume formate without electricity production and their proliferation within the MFC was thought to be due to the microaerobic conditions in the anodic chamber of the device (Ha *et al*., 2007). The microaerobic nature of *Arcobacter spp*. growth in this system was found by supplying the system with azide (an aerobic growth inhibitor) and observing a reduction in the decrease of

COD (Ha *et al*., 2007). Therefore while *A. butzleri* ED-1 and *Arcobacter* L were not the first *Arcobacter spp*. to be isolated from an MFC they are the first *Arcobacter spp*. found to be capable of electrogenesis.

The bacteria were isolated from a horizontal multi-electrode MFC and were cultured in a phosphate buffered basal medium containing 1.0 COD/L of acetate and fed on a weekly basis to maintain this concentration of acetate. It was inoculated with 0.5 L of marine sediment taken from a depth of 3 m from Cramond, Edinburgh (Fedorovich *et al*., 2009). After 4 weeks of continuous operation samples of the anode and planktonic phase of the anode chamber were taken and analysed by DGGE (Fedorovitch *et al*., 2009). The DGGE results revealed the presence of two bands not present in the original marine sediment and subsequent analysis and 16S rRNA sequencing revealed these bands to be *A. butzleri* ED-1 (on the anode) and *Arcobacter* L (in the planktonic phase). *A. butzleri* ED-1 and *Arcobacter* L made up the majority of the population on the anode and in the planktonic phase respectively. The *Arcobacter spp*. was isolated using *Arcobacter* selective medium i.e. Houf blood agar (Houf *et al*., 2001, Fedorovich *et al*., 2009). The bacteria were studied in pure culture in batch experiments using half MFCs (see chapter 2) and in microtitre plates where they were shown to be capable of using acetate as their sole carbon source and capable of generating highly negative redox potentials in pure culture, with *A. butzleri* ED-1 cultures displaying greater electrogenic ability than *Arcobacter* L cultures (Fedorovitch *et al*., 2009). These results were of considerable interest as they were the first examples of ε proteobacteria performing electrogenesis and show that electrogenic ability is more widespread than previously predicted (Fedorovich *et*

al., 2009). Subsequent genome sequencing of A. *butzleri* ED-1 and *Arcobacter* L showed that *A. butzleri* ED-1 lacked homologous proteins to those associated with pathways of electrogenesis in *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 (Toh *et al*., 2011). Therefore it was possible that *A. butzleri* ED-1 was performing electrogenesis by a very different mechanism than those previously characterised. It was decided to study how these two species interact with the anode of MFCs and analyse their electrogenic mechanisms (the aims of the study are discussed more fully in section 1.13).

These bacteria are examples of *Arcobacter spp*. fulfilling a particular niche; therefore it is important to understand them in the greater context of the *Arcobacter* genus which is discussed in section 1.8.

1.8 The genus *Arcobacter*

Arcobacter are Gram negative, non-fermentative, non-spore forming rods typically 0.5-3 μm long and 0.2-0.9 μm wide (Ho *et al.*, 2006). The bacteria are typically S shaped and are highly motile by means of single, unsheathed polar flagella at either one or both ends of the cell (Ho *et al*., 2006). The morphology and flagella are shown in the electron micrographs in Figure 1.12.

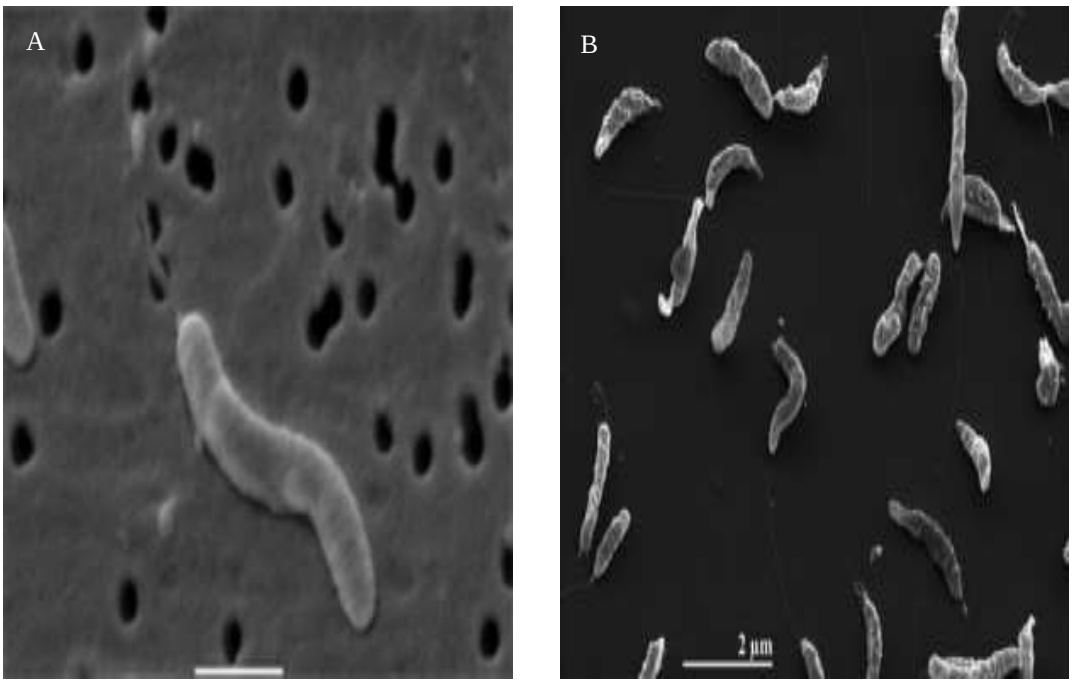


Figure 1.12 adapted from Houf *et al*., 2009 and Pati *et al*., 2010. Image A) a scanning electron micrograph of *Arcobacter therius*, a species of *Arcobacter* isolated from pigs and ducks. The bar represents 1 µm. image B) *Arcobacter nitrofigilis*, a nitrogen fixing species of *Arcobacter* that lives in the nodules of the salt marsh plan *Spartina alterniflora*. Both *Arcobacter* species have a single polar flagellum and are s shaped. Unlike other *Campylobacteraceae*, *Arcobacter spp*. are found in a wide range of environments as highlighted by the radically different environments of the two species shown here.

Arcobacter are member of the *Campylobacteraceae* family (Vandamme *et al*., 1991). This contains the genus *Campylobacter*, *Helicobacter* and *Wolinella* (Ursing *et al*., 1994) which is in turn a member of the ε proteobacteria subdivision. *Arcobacter* are distinct from other *Campylobacteraceae* as they are capable of aerobic growth and can grow in the temperature range 15 °C-42 °C (Van Driessche *et al*., 2003). Aerotolerance and a wide range of growth temperatures mean that *Arcobacter spp*. are found in a number of different environments and in association with humans and

animals. This is atypical of most *Campylobacteraceae*, which are usually just found in association with humans and/or animals (Collado and Figueras 2011). *Arcobacter spp*. have been found in a wide range of environments including deep sea hydrothermal vents, hydrocarbon contaminated seawater, shellfish, abattoirs, human and animal faeces, tube worms, anaerobic sludge and a petroleum reservoir to name but a few (Miller *et al*., 2007).

1.9 The different *Arcobacter* species

There are currently 12 different species of *Arcobacter*, some of which are found associated with human and animals, some of which are found free living in the environment and some which are found in both. The following pylogenetic tree shows the relationship of different *Arcobacter spp*.

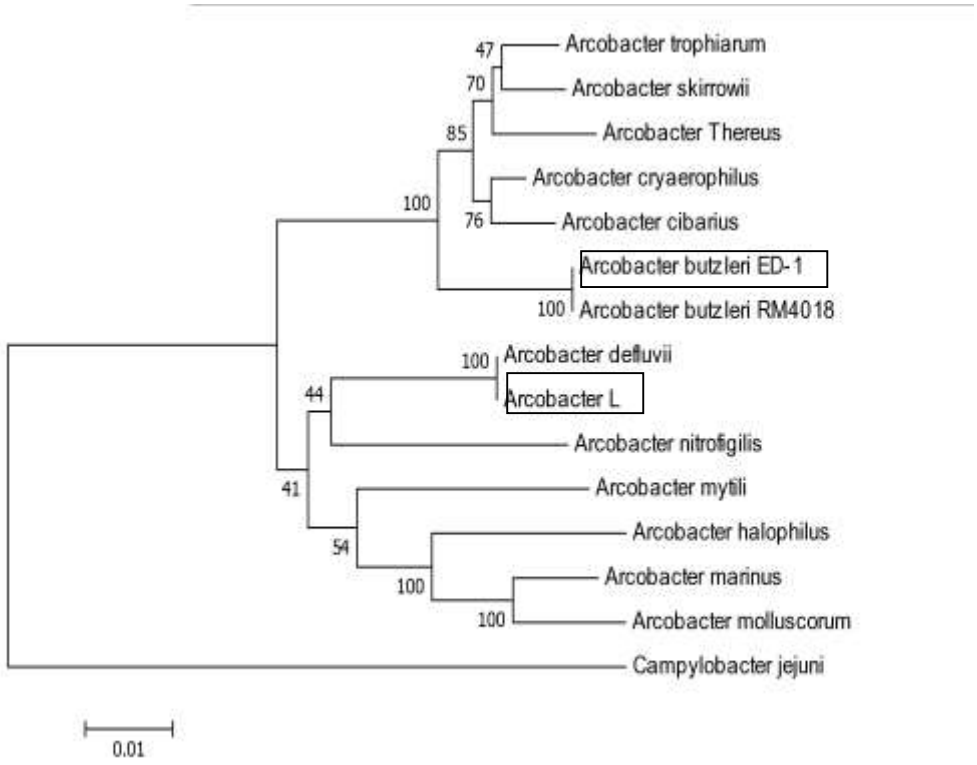


Figure 1.13 shows a neighbour-joining tree displaying the relationships between the different species of *Arcobacter* (tree constructed by 100* bootstrapping). The tree was constructed using the complete sequence of 16S rRNA gene (1.5 kb) from each species. The electrogenic species (*A. butzleri* ED-1 and *Arcobacter* L) are shown in boxes. There is a close relationship between *Arcobacter* L and *A. defluvii* which suggests that *Arcobacter* L is a strain of *A. defluvii*. The neighbour joining tree was constructed by use of the molecular evolutionary genetic analysis software (MEGA) for constructing phylogenetic trees (www.megasoftware. net 2012)

There are considerable differences between each species of *Arcobacter* but also distinct similarities; Table 1.3 compares and contrasts the characteristics of the different species of *Arcobacter.*

Species	%G+C content	Growth at 10°C	Growth at 30°C	Growth at 37°C	Catalase positive	Oxidase positive	Urase positive	Reduces nitrate	Isolated from	Notes
A. thereius	28.5	-	+	-	+	-	+	+	Pigs and Ducks	Grows on minimal media
A trophiarum	28.5	-	+	-	+	-	+	+	Fattening pigs	
A.skirrowii	29-30	-	+	+	+	-	?	+	Aborted porcine foetus and porcine faeces	
A. butzleri	27	-	+	+	+	-	+	+	Porcine and bovine foetuses, spoiled meat, a variety of environmental sources	
A.cryaerophilus	30	-	+		+	-	?	-	Aborted porcine and bovine foetuses and faeces	
A. cibarius	26.8-27.3	-	+	+	+	+	-	+	Chicken broiler carcasses	Displays a "stubber" morphology then most *Arcobscter* spp., thrives under aerobic conditions
A. nitrofigilis	28	+	+	+	+	-	-	+	Root nodules of *Spartina alterniflora*	Capable of nitrogen fixation, obligate microaerobe
A. defluvii	?	?	?	?	?	?	?	?	Isolated from sewage	
A. mytili	26.9	--	+	+	-+	+	--	+	Recovered from Muscles of the *Mytilus* genus	Is indoxyl-acetate hydrolysis negative,
A. halophilus	35	-	+	+	+	+	-	+	Hypersaline lagoon	Obligate halophile
A. marinus	28	-	+	+	+	-	-	+	East sea, Korea	
A.molluscorum	≈30	-	+	-	+	-	-	+	Muscles and oysters	

Table 1. 3 compares the different qualities of different *Arcobacter* species. The table compares the G+C content of different *Arcobacter* species, growth at 10°C, 30°C and 37°C and the ability of the bacteria to produce catalase, oxidase, urease and nitrate reductase. The table also details where the different species of *Arcobacter* were isolated from as well as any additional information. Table 1.3 shows that *Arcobacter* are found in a wide range of environments and all have catalase activity (which explains their aerotolerant nature). Species able to thrive under aerobic conditions such as *A. cibarius* also possessed oxidase activity. *Arcobacter* associated with animals or humans tend to possess an ability to grow at higher temperatures than the species found free living in the environment.

Given the varied nature of the *Arcobacter* genus it is unsurprising that a variety of *Arcobacter* species have been found to be or at least implicated to be human and animal pathogens.

1.10 *Arcobacter* as an animal and human pathogen

Arcobacter spp. have been found or at least implicated to be the pathogenic agent in a number of cases involving human and animal disease in recent years (Ho *et al*., 2006). While the role of *A. butzleri* as a pathogen is not the focus of this project, *Arcobacter* species as pathogens must be discussed in order to understand the genus as a whole.

1.10.1 *Arcobacter spp*. as an animal pathogen

A variety of different *Arcobacter* have been found in the faeces of healthy pigs (Hume *et al.,* 2001) and cattle with detection in sheep and horses also being reported (Kabeya *et al.,* 2003, Van driessche *et al.,* 2003). In these studies the most commonly detected bacterium was *A. butzleri* followed by *A. cryaerophilus* although *A. skirrowii* was found in certain isolates Kabeya *et al,* 2003, Van driessche *et al.,* 2003). All the animals were clinically healthy displaying none of the typical symptoms of *Arcobacter* infection such as fever, watery diarrhoea or vomiting (Kabeya *et al,* 2003, Van driessche *et al,* 2003). *Arcobacter spp*. has also been isolated from foods of animal origin and from the slaughterhouse carcasses of cattle, chickens and pigs (Ho *et al*., 2006). In certain studies the bacteria were found in 70%

of chicken samples and >20% of pork and beef samples (Rivas *et al*., 2004) with numbers of *Arcobacter* being calculated at several thousand bacteria per gram of sample (Houf *et al*., 2002). In these studies *A. butzleri* and *A. cryaerophilus* were the most common species isolated although *A. skirrowii* was also found (Houf *et al*., 2002). These studies suggest that these species of *Arcobacter* are commensal bacteria in these animals.

A. butzleri, A. cryaerophilus and *A. skirrowii* have been found to be the causative agents or implicated to be the causative agents in a number of animal pathologies. *Arcobacter* were first isolated from aborted porcine and bovine foetuses (Ellis *et al.,* 1997, Ellis *et al.,* 1978) where they were later found to be *A. butzleri, A. cryaerophilus and A. skirrowii* (Vandamme *et al.,* 1991, Vandamme *et al.,* 1992). Since then these species of *Arcobacter* have been found to be associated with abortions, infertility and chronic still born problems (Schroeder-Tucker *et al.,* 1996). *Arcobacter* have also been found to be associated with diarrhoeal pathologies in animals; strains of *A. butzleri* and *A. skirrowii* have been isolated from strains of sheep and cattle afflicted with diarrhoea and haemorrhagic colitis (Vandamme *et al.,* 1992). *A.butzleri* has also been isolated from the non-human primate *Macaca mulatta* (Anderson *et al.,* 1993). The primates were found to be suffering from diarrhoeal disease with associated chronic, active colitis, which coupled with ribotyping, suggests *A. butzleri* is endemic in the primate population (Anderson *et al.,* 1993).

This suggests that the bacterium acts as an opportunistic pathogen in these animals with the naturally occurring *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* serve as a reservoir for infection (Lehner *et al*., 2005).

1.10.2 *Arcobacter spp*. as a human pathogen

Arcobacter are an emerging human pathogen and have been associated with a number of cases of diarrhoeal disease and enteritis (Ho *et al.*, 2006). *A. butzleri* and to a lesser extent *A. cryaerophilus* have shown an association with stool samples from patients suffering from enteritis (Vandenberg *et al*, 2004). *A. butzleri* infection is typically associated with persistent watery diarrhoea although nausea, vomiting and fever are all associated wi*th A. butzleri* infection (Vandenberg *et al.*, 2004).). *Arcobacter spp*. have subsequently found to be the causative agent in a number of incidents of diarrhoeal disease; *A. butzleri* was found to be the causative agent in 8% of cases travellers' diarrhoea in a study performed in 2010 (Jiang *et al*., 2010) and *A. butzleri* was isolated from the stools of 0.4 % of patients suffering from infectious gastroenteritis (De Boer *et al*., 2013). Therefore it is increasingly clear that *Arcobacter spp*. particularly *A. butzleri* are an emerging human pathogen or opportunistic pathogen associated with diarrhoeal disease.

As well as diarrhoea, *Arcobacter* has been found to be associated with a number of other pathologies. *A. butzleri* was found to be the causative agent in a case of acute gangrenous appendicitis (Lau *et al.*, 2002) and *A. cryaerophilus* was found as the

causative agent in a case of bacteraemia in a road traffic victim who fell into mud (Woo *et al,* 2001). The latter case is of some interest as evidence suggests that the bacteria entered the blood stream through the respiratory system (Woo *et al.,* 2001), which is unusual as the route of *Arcobacter* infection is thought to be through faecal-oral transmission or eating/ drinking contaminated food or water. *A. butzleri* was also the causative organism in a case of neonatal septicaemia in which the infection was contracted in *utero,* although the mother showed no signs of infection (On *et al.*, 1995). This case correlates with the isolation of *Arcobacter spp*. from aborted animal foetus, which suggests the bacteria can cause prenatal and neonatal pathology.

Like all pathogens *Arcobacter spp*. processes virulence factors, which are discussed below.

1.10.3 The virulence factors of pathogenic *Arcobacter spp*.

Genome sequencing of *A. butzleri* RM4018 has revealed several homologs to *C. jejuni* virulence factors (Miller *et al*., 2007). The bacterium contains homologs to the fibronectin binding proteins CadF and Cj1349, the invasin protein CiaB, the phospholipase PldA, the heamolysin TlyA and the virulence factor MviN (Miller *et al*., 2007). The bacterium also encodes for an IrGA homolog, which is a virulence factor in pathogenic *E. coli* and *Vibrio cholera* (Miller *et al*., 2007).

Therefore it is unsurprising that *Arcobacter spp*. have been shown to be capable of adhering to and invading various cell lines (Carbone *et al*., 2003); in this study various strains of *A. butzleri* were shown to adhere to HeLa nd Hep-2 cell lines .Figure 1.14 shows *A. butzleri* adhering to Hep-2 epithelial cells.

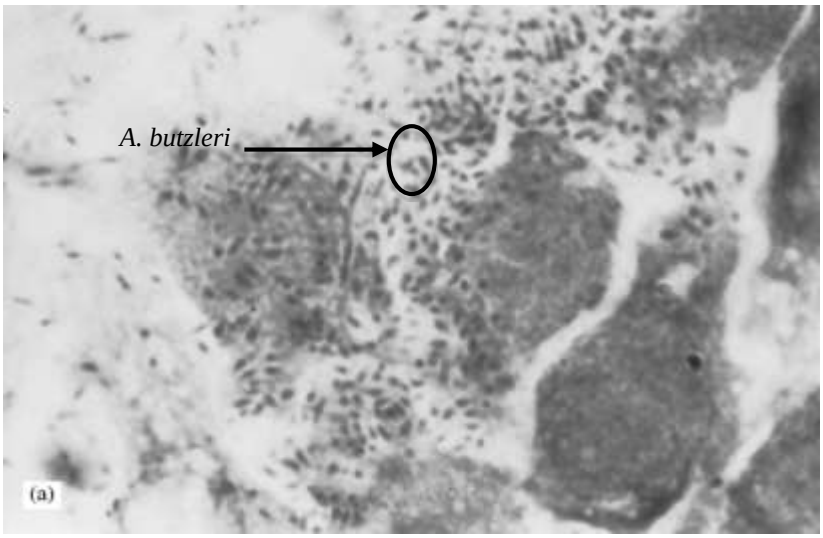


Figure 1.14 adapted from Carbone *et al*., 2003; it shows a light micrograph (1000* magnification, scale bars not shown in original publication) of *A. butzleri* (circled) adhered to Hep-2 cells. The *A. butzleri* also secreted toxic factors that had a similar effect to the cytolethal distending toxin of *C. jejuni*, despite possessing no apparent homolog to this protein (Johnson and Murano 2002, Miller *et al*., 2007).

The best characterised of the *Arcobacter* adhesins is CadF, which is discussed in further detail in section 1.10.3.1

1.10.3.1 CadF

CadF is a 37 kDa outer membrane protein found in *C. jejuni* (Konkel *et al*. 1997). It is important in the adhesion of the bacterium to fibronectin in the extracellular matrix (Konkel *et al*. 1997). The binding of CadF to fibronectin is mediated by a 4 amino acid domain (AA134-137) consisting of Phenylalanine-arganine- leucine- serine (Konkel *et a*l. 2005) as shown when Phe-Arg-Leu was changed to Ala-Ala-Gly which resulted in a 91% decrease in fibronectin binding of the protein (Konkel *et al*. 2005).

The binding of fibronectin by CadF is an important precursor to cell invasion by *Campylobacter*, triggering a cell-signalling pathway involving paxillin which results in actin rearrangement leading to *C. jejuni* being internalized and subsequently able to invade epithelial cells (Monteville *et al*. 2003). Since *Arcobacter spp*. has also shown a capacity for invading cells, the CadF homolog presumably acts in a similar fashion.

Despite the presence of CadF the bacterium lacks homologs for other *Campylobacter spp.* adhesins such as JlpA and Peb1 (Miller *et al*., 2007). It must be noted that the bacterium contains genes for several other adhesins or potential adhesins such as an IrgA homolog (Miller *et al*., 2007) which was found to be a virulence factor in uropathogenic *E. coli*, with *irgA*$^{-}$ mutants being severely impeded in their ability to colonise T 24 human uroepithelial cells (Johnson *et al*., 2005).

As well as adhering to and invading cells *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have also displayed cytotoxic capabilities. The cytotoxic capabilities of these *Arcobacter* species are discussed in section 1.10.3.2.

1.10.3.2 Cytotoxic capabilities of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*

Studies have shown that *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* isolated from meat samples displayed toxicity against vero cells with elongation and vacuole formation being associated with 38% of these isolates (Lopez *et al*., 2003). Other studies have shown that *Arcobacter* exhibit similar toxic effects on HeLa and Int407 cells (Johnson and Murano 2002). This toxic effect is similar to that observed with *C. jejuni* which produces cytolethal distending toxin. Therefore the presence of homologs to *C. jejuni* cytolethal distending toxin was studied in a variety of strains of *Arcobacter* (Johnson and Murano 2002) but no homologues were found. Genome sequencing of *A. butzleri* RM4018 confirmed that *A. butzleri* RM4018 contained no genes homologous to cytolethal distending toxin (Miller *et al.*, 2007). Therefore it is likely that pathogenic *Arcobacter* produce a toxin distinct from *C. jejuni* cytolethal distending toxin that nevertheless has a similar function. The exact nature of this toxin is as yet unknown.

The above cases and the presence of virulence factors suggest that certain species of *Arcobacter* are opportunistic pathogens or pathogens for humans and animals that have pathology similar to *Campylobacter* infection (Miller *et al*., 2007).

Arcobacter are more than just a pathogen, they have been found in a number of different environments, which in the context of *Arcobacter* in MFCs is of greater importance. *Arcobacter* as an environmental organism is discussed in section 1.11.

1.11 *Arcobacter* as an environmental organism

1.11.1 *Arcobacter* and dissimilatory metal reduction

Arcobacter have been found in high numbers in environments where dissimilatory metal reduction is taking place. *Arcobacter* were first found to be capable of dissimilatory metal reduction during a study into Black Sea shelf sediment where dissimilatory manganese reduction was the primary anaerobic process (Thamdrup *et al*., 2000). In this study *Arcobacter spp*. were the only species detected by the use of 16S clone libraries (Thamdrup *et al*., 2000). It is thought that *Arcobacter* species anaerobically oxidise acetate and use Mn^{4+} as an insoluble terminal electron acceptor within this particular environment (Thamdrup *et al*., 2000). More recent studies have also shown that *Arcobacter spp*. are found in high numbers in anaerobic environments where high levels of acetate-dependent manganese reduction takes place (Vandieken *et al*., 2012). This study also revealed high numbers of bacteria from the *Colwellia* and *Oceanospirillaceae* taxa, which suggests that the bacteria involved in dissimilatory manganese reduction are quite distinct to those typically found to be involved in iron reduction (Vandieken *et al*., 2012).

Arcobacter were also identified in cultures designed to isolate Fe^{3+} reducing organisms. The set up used a medium containing sugar beet molasses as a carbon source, Fe^{3+} as electron acceptor and lake sediment as an inoculant (Sikora et *al*., 2011). *Arcobacter spp*. (with a 99% similarity to *A. butzleri*) was found to comprise 25%-35% of all isolated bacteria with *Shewanella* being the most abundant isolate (Sikora et *al*., 2011). Unpublished results (see chapter 3) have also suggested that *A. butzleri* ED-1 is capable of reducing Fe^{3+}. Therefore one possible reason for the proliferation of *Shewanella* in this system is that *Shewanella* is better adapted to the substrate present. Another reason is that *Arcobacter spp*. are less adapted to using Fe^{3+} as a terminal electron acceptor than *Shewanella* and are therefore out competed in the system.

These results suggest that *Arcobacter spp*. play an important role in dissimilatory Mn^{4+} reduction and while they are capable of reducing iron they do not play as significant a role in this process as *Shewanella* and *Geobacter spp*. do. One potential reason for this is that their pathways of extracellular electron transfer are possibly quite different from those characterised in *Geobacter* and *Shewanella spp*. However without further study this is unknown.

1. 11.2 *Arcobacter* species in biofilms on abiotic surfaces

Biofilm formation is an important part of electrogenesis in a number of species and since *A. butzleri* ED-1 was isolated from the anode of an MFC (Fedorovich *et al*.,

2009) it is important to understand the ability of *Arcobacter* to interact with other abiotic surfaces.

A. butzleri was found to be capable of attaching to a number of abiotic surfaces, including stainless steel, copper and plastic in water distribution pipe surfaces (Assanta *et al*., 2002). Adhesion was tested at temperatures of 4°C and 20°C, and bacteria were found to adhere more strongly at 20 °C (Assanta *et al*., 2002). The bacteria were found to produce extracellular fibrils, especially after 72 h of contact and particularly on stainless steel surfaces (Assanta *et al*., 2002). Further studies have shown that *A. butzleri* can form biofilms on abiotic surfaces at temperatures ranging from 5 -37 °C, with biofilm formation being stronger at higher temperatures (Kjeldgaard *et al*., 2009). Stronger biofilm formation at temperatures above 10 °C is unsurprising as the minimal growth temperature for *Arcobacter spp*. is 10 -15 °C.

Arcobacter spp. have also been found as a component of sulfide oxidising, nitrate reducing anaerobic biofilms in wastewater (Lomas *et al*., 2007). Genomic analysis has shown that *Arcobacter* are capable of sulphide oxidation (Miller *et al*., 2007) possessing SOX genes and also possess functional nitrate reductase (Miller *et al*., 2007 and see Table 1.3). *Arcobacter spp*. were also found to form a biofilm in the recirculation systems for the storage the European lobster *Homarus gammarus* (Welsh *et al*., 2011). Other studies have found *Arcobacter* to be present in biofilms in other sulphide rich environments (Macalady *et al*., 2006). In this particular study they were found to be a part of limestone corroding biofilms in the Frassii cave system, Italy (Macalady *et al*., 2006). It must be noted that *Arcobacter* did not

comprise the majority of species in this environment (*Desulfocapsa spp.* formed the majority) although they did form the majority of ε proteobacteria detected (Macalady *et al*., 2006). Therefore it is possible that *Arcobacter* play a role in sulfur cycling within these environments.

Therefore it is clear that *Arcobacter spp.* are capable of forming biofilms on a number of abiotic surfaces and can sometimes play a role in an important ecological function.

1.11.3 *Arcobacter* as a waterborne organism

Species of *Arcobacter* have been isolated from a variety of fresh and salt-water sources. *Arcobacter* species were isolated from crude oil contaminated sub-Antarctic seawater, although they were not found in non-contaminated seawater from the same location (Prabagaran *et al*., 2006). The *Arcobacter* were presumably present in low numbers in the seawater normally but enriched by the presence of crude oil (Prabagaran *et al*., 2006). Here the *Arcobacter* presumably oxidised sulphide present (Wrisen *et al*., 2002). The lack of *Arcobacter* in uncontaminated seawater suggest that *Arcobacter* are not present in high numbers in sea water unless enriched by a change in the environment such as the contamination by hydrocarbons alters the conditions to allow them to proliferate.

Arcobacter species have also been detected in a variety of fresh water sources. *Arcobacter* with a 97-99% similarity to *Arcobacter cryaerophilus* were isolated from

stagnant dairy wastewater lagoons and *Arcobacter* with a 96-98% similar to *Arcobacter cibarius* were found in circulated wastewater (McGarvey *et al*., 2005). The circulation of wastewater would increase O_2 levels that would favour *A. cibarius*, as it is more aerotolerant than *A. cryaerophilus* due to the presence of an oxidase (see Table 1.3). *A. butzleri* has also been isolated from groundwater (Rice *et al*., 1999) and from various points along the Llobregat River catchment (Catalonia, Spain) where it was typically associated with faecal contaminated locations, where it composed 90% of the population (Collado *et al*., 2010). *A. cryaerophilus* and *A. skirrowii* were also isolated from this location comprising 18% and 0.3% of the population respectively (Collado *et al*., 2010).

The above examples show the versatility of the different *Arcobacter spp*. and their ability to live as commensal organism in various animals, act as a pathogen/ opportunistic pathogen and as a free living organism in a variety of different environments. The versatility of *Arcobacter* is due in part to the versatility of its metabolism.

1.12 The metabolism of *A. butzleri*

The availability of genome sequences for both *A. butzleri* RM4018 and *A. butzleri* ED-1 has led to the study of the bacterium's central metabolism in considerable detail.

1.12.1 The TCA cycle, methylcitrate cycle and citramalate cycle of *A. butzleri*

A. butzleri RM4018 and *A. butzleri* ED-1 encode for a nearly complete TCA cycle although the bacteria lack the genes encoding for succinyl CoA synthatase and succinate dehydrogenease (Miller *et al*., 2007, Ler 2009). The bacterium does encode for a fumarate reductase that favours the reduction of succinate to fumarate, which suggests that the bacterium thrives under anaerobic conditions (Ler 2009). The bacteria also contain genes for citrate synthase and isocitrate dehydrogenase, which suggests that the TCA cycle operates in an oxidative direction at least from oxaloacetate to succinyl-CoA (Ler 2009).

The lack of succinyl CoA synthetase and succinate dehydrogenase raises the question how does the TCA cycle operate in an oxidative direction as certain key intermediates would surely not be replenished? The answer to this may lie in the methyl citrate and citramalate of the bacterium.

Genomic analysis suggests that *A. butzleri* possesses a functioning methylcitrate cycle (Miller *et al*., 2007, Ler 2009). The bacterium has been found to encode for several key enzymes of the methylcitrate cycle; methylcitrate synthase , methylcitrate dehydratase and methylisocitrate lyase (Miller *et al*., 2007, Ler 2009), The methyl-citrate cycle produces fumarate, malate, oxaloacetate and pyruvate, which it shares with the TCA cycle, effectively forming a closed loop spanning succinate to oxaloacetate (Ler 2009). Therefore it is thought that the methyl citrate cycle is important in allowing the TCA cycle of *A. butzleri* ED-1 to operate in an oxidative direction despite lacking succinyl CoA synthetase and succinate dehydrogenase, as it replenishes several intermediates that would otherwise not be replenished due to this lack of enzymes and therefore it essentially "bridges the gap" (Miller *et al*., 2007, Ler 2009). This is illustrated in Figure 1.15.

In addition to a functioning methylcitrate cycle, *A. butzleri* has also been found to encode many genes that would make up a functional citramalate cycle (Miller *et al*., 2007, Ler 2009). The bacterium has been found to encode for citrate lyase, AMP dependent synthatase and ligase, all of which are key components of a functioning citramalate cycle (Miller *et al*., 2007, Ler 2009). The citramalate cycle also generates glyoxylate, which is important as the bacterium lacks a functioning glyoxylate cycle and therefore the citramalate cycle supplies an important intermediate in the metabolism of acetate (Ler 2009). In addition the citramalate cycle generates pyruvate and malate that feed into the TCA cycle and therefore it also plays a secondary role in "bridging the gap" in the TCA cycle.

The interactions of the TCA cycle, methylcitrate cycle and citramalate cycle are illustrated in Figure 1.15.

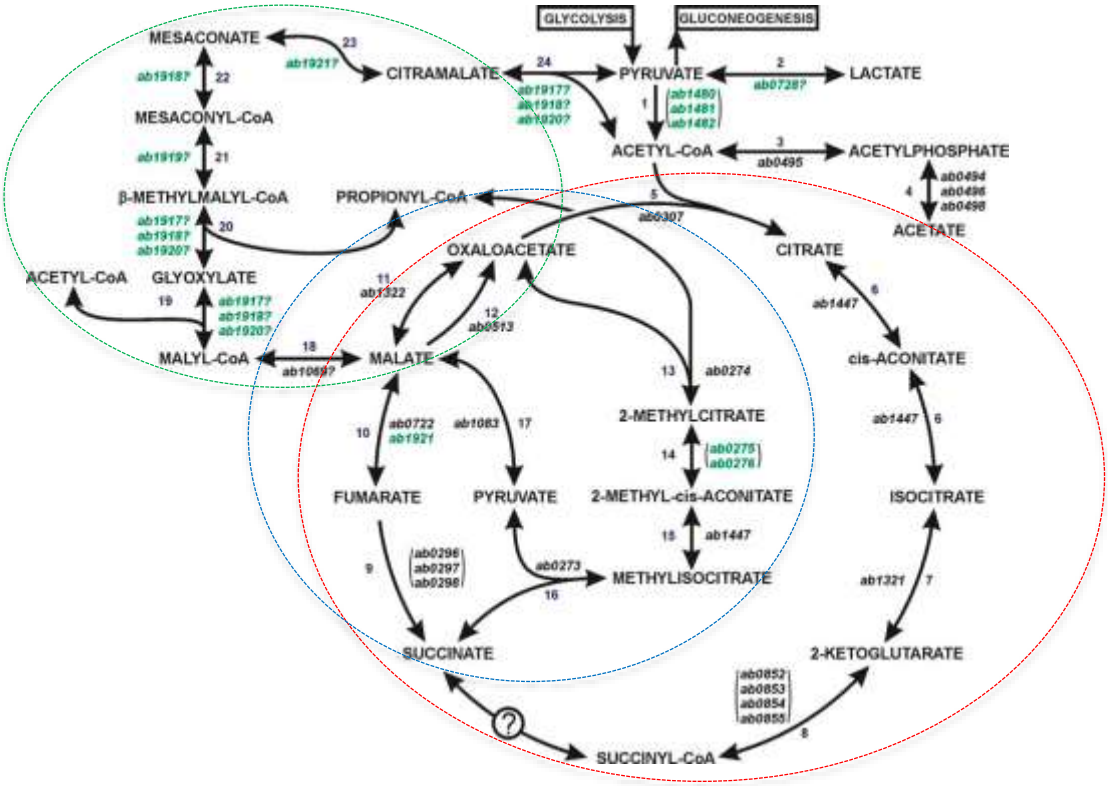


Figure 1.15 adapted from Miller *et al*., 2007. It shows the TCA cycle (red) and how it interacts with the methylcitrate cycle (blue). It shows the oxaloacetate and malate from the methylcitrate cycle forming a loop which bypasses the succinate to succinyl-Co A stage which allows the TCA cycle to be fully functional in an oxidative direction despite the gaps. The citramalate cycle (green) generates glyoxylate and interacts with the TCA cycle at the malate and pyruvate points.

The generation of glyoxylate by the citramalate cycle is the only source of glyoxylate for the bacteria since the bacterium lacks the genes required for a functioning glyoxylate cycle (Miller *et al*., 2007, Ler 2009). It is therefore important in acetate metabolism since glyoxylate is an important component of that process. The different carbon sources *Arcobacter* is capable of using are discussed in section 1.11.2

1.11.2 Carbon source usage by *A. butzleri*

The lack of 6-phosphofructokinase, hexokinase, glucokinase and a glucose uptake system means that *A. butzleri* lacks a functioning glycolysis pathway and is therefore unable to utilise glucose or other similar sugars as a carbon source (Miller *et al*., 2007, Ler 2009). The bacterium is capable of using a number of organic acids as carbon sources. *A. butzleri* is capable of using acetate, lactate, malate, propanoate and succinate as a carbon source (Miller *et al*., 2007, Ler 2009). Figures 1.16-1.17 illustrates how the bacterium uses acetate and lactate and table 1.4 gives the energy derived from each of the predicted carbon sources.

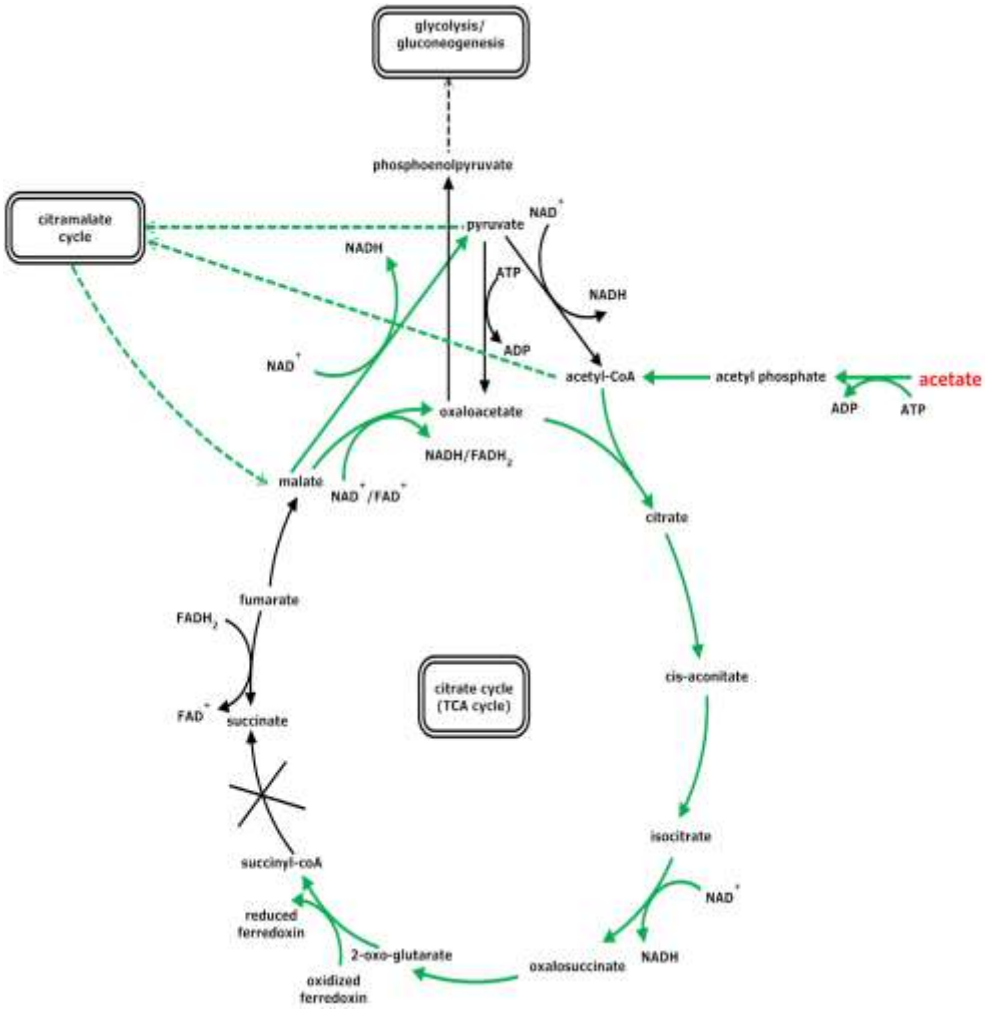


Figure 1.16 adapted from Ler 2009. It shows the proposed path of acetate metabolism by *A. butzleri* ED-1 Red shows the entry compound and green arrows is the path traversed by the molecule. Acetate is converted into acetyl phosphate and then into two molecules of acetyl coA. One molecule of acetyl CoA directly enters the TCA cycle and traverses it as far as succinyl CoA. The other molecule of acetyl CoA enters the citramalate cycle and then re-enters the TCA cycle as malate. This malate is then converted into oxaloacetate; thereby regenerating the oxaloacetate required for acetate metabolism. Therefore two molecules of acetate generate 1 $FADH_2$, 2 NADH and 1 reduced ferredoxin.

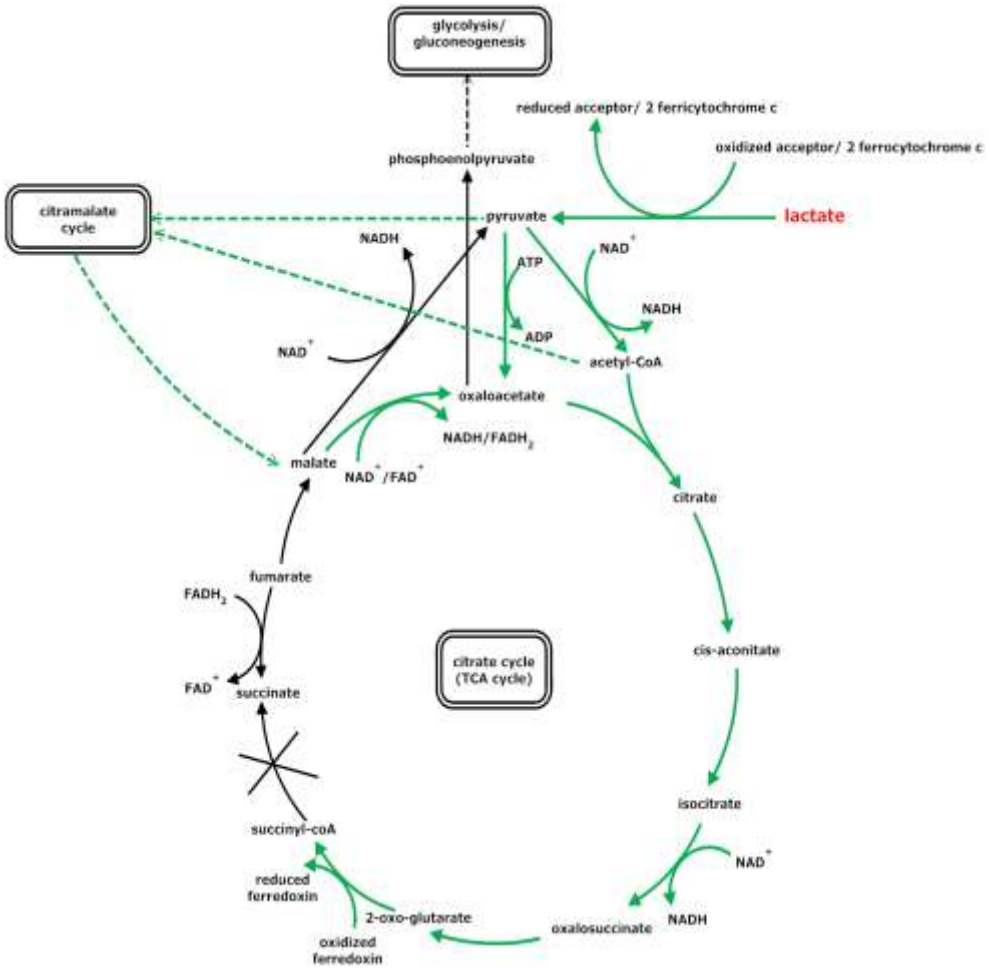


Figure 1.17 adapted from Ler 2009. It shows the proposed path of lactate metabolism (green arrows) in *A. butzleri* ED-1. Lactate is converted into pyruvate and acetyl-coA which then traverse the TCA cycle to succinyl coA. Other molecules of pyruvate and acetyl-CoA enter the citramalate cycle and then re-enter the TCA cycle as malate which is then converted to oxaloacetate, thus replenishing oxaloacetate. This generates 7$FADH_2$, 6NADH and 1 reduced ferredoxin. The increase energy generated explains why *A. butzleri* exhibits stronger growth on lactate rather than acetate.

Carbon source	Energy carrying units yielded
Acetate	1 FADH2, 2 NADH and 1 reduced ferredoxin
Lactate	2 NADH, 2 reduced acceptor, 1 reduced ferredoxin and 4 reduced ferrocytochrome c
Malate	4 NADH, 1 $FADH_2$ and 1 reduced ferredoxin
Propanoate	7 $FADH_2$, 6 NADH and 1 reduced ferredoxin
Succinate	3 $FADH_2$, 4 NADH and 1 reduced ferredoxin

.

Table 1.4 adapted from Ler 2009 shows how many different energy carrying units are yielded by carbon sources commonly used by *A. butzleri* ED-1. It shows that *A. butzleri* ED-1 theoretically generates the most energy carrying units from propanoate and therefore should exhibit the strongest growth on propanoate.

The metabolic reconstruction shows that *A. butzleri* is capable of potentially utilising a wide variety of organic acids, which in the context of MFCs makes them useful for treating wastewaters and other feed stocks that contain a high amount of organic acids. The ability of the bacterium to use these organic acids as a carbon source was validated by experimental evidence, which is discussed in chapter 3.

While this is of interest in terms of practical applications of *A. butzleri* in MFCs it does not cover electron transfer and what pathways of electron transfer may be involved in extracellular electron transfer in *Arcobacter*.

1.11.3 Electron transfer pathways of A*. butzleri*

A. butzleri encodes for a full set of genes for aerobic/ microaerobic respiration including NADH: quinone oxidoreductase, ubiquinol cytochromes c oxidase, ferredoxin, cytochromes *bd* oxidase, cytochromes c oxidase (*cbb*3-type) and F1/F0 ATPase (Miller *et al*., 2007, Ler 2009). The bacterium also encodes for a number of genes important in anaerobic respiration including fumarate reductase, and nitrate reductases, found in an operon analogous to the *nap* operon found in *Campylobacter* species (Miller *et al*., 2007, Ler 2009). The genome has also been found to contain the C type cytochromes TorA and TorC allowing it to utilise TMAO as an electron acceptor (Miller *et al*., 2007, Ler 2009). The bacterium also encodes for the reductase DmsA allowing it to use DMSO as a terminal electron acceptor and a b type nitrite/ nitric oxide reductase has also been identified (Miller *et al*., 2007, Ler 2009). This shows that *A. butzleri* is capable of using a wide variety of electron acceptors. These different pathways of electron transfer in *A. butzleri* ED-1 are illustrated in Figure 1.18.

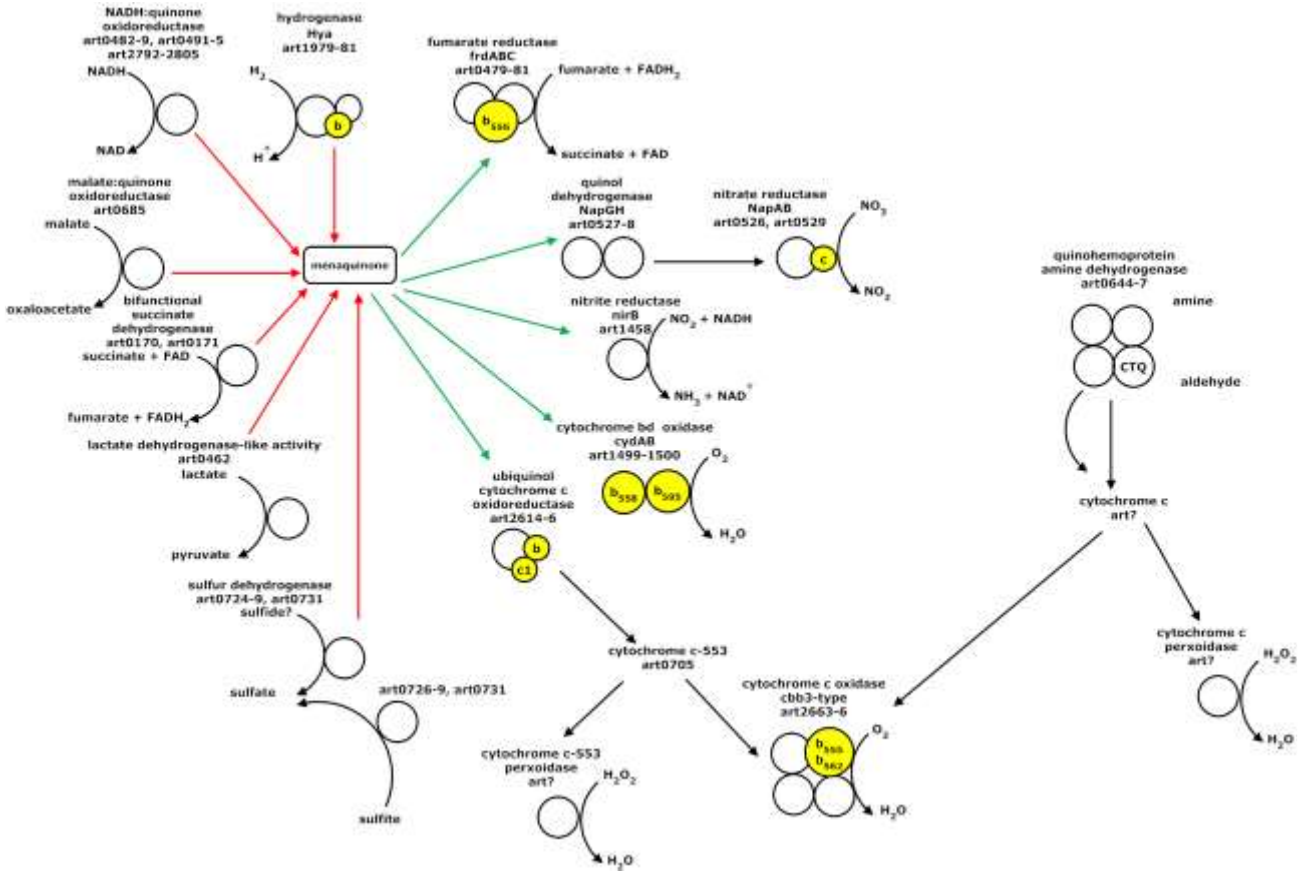


Figure 1.18 adapted from Ler 2009 and shows the electron transfer pathway in *A. butzleri* ED-1. Blue arrows show electrons being transferred to a central menaquinone (MQ) pool and red arrows show the electrons being transferred away from the MQ pool. The diagram shows electrons being transferred down a pathway for aerobic respiration, which ends with electrons being transferred to oxygen by cytochrome c oxidase.

The electron transfer pathways of *A. butzleri* ED-1, *A. butzleri* RM4018 and *Arcobacter* L are similar to each other although there are several key differences. *A. butzleri* ED-1 lacks cytochrome bd oxidase and the sulphur oxidase (SOX) system

genes (Ler 2009). *Arcobacter* L encodes for the SOX system, but lacks TorC and is unable to use formate as an electron donor to the menaquinone pool due to a lack of formate dehydrogenase.

These electron transfer pathways do not provide any insight into how *A. butzleri* ED-1 performs extracellular electron transfer as all of the pathways are for soluble electron acceptors, which have been shown to use different electron transport chains then insoluble electron acceptors i.e. in *Geobacter* bacteria capable of extracellular electron transfer can still grow using fumarate as a terminal electron acceptor (Regurea *et al*., 2005). Therefore while a vital tool the genomic sequence does not provide any immediate insight into how *A. butzleri* is capable of electrogenesis.

1.13 The aims of the project

Based on the information discussed throughout the introduction this project has two key aims; these are discussed in section 1.13.1 and 1.13.2.

1.13.1 To elucidate the components of extracellular electron transfer in *A. butzleri* ED-1

A. butzleri ED-1 lacks outer membrane cytochromes and pili homologous to those characterised as important for extracellular electron transfer in *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1. It is unknown whether any of the proteins encoded for by *A. butzleri* ED-1 fulfil any analogous role. It was therefore possible that *A. butzleri* ED-1 performs electrogenesis in a significantly different

fashion from *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1. If *A. butzleri* ED-1 performed electrogenesis by similar methods to *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 the up-regulation of electron transporter proteins and adhesins (to function as nanowires) would be expected at the anode as previously observed (Nevin *et al*., 2009, Rosembaum *et al*., 2012). If the methods of electrogenesis were different it may be expected to see up-regulation of very different proteins at the anode.

It was decided to perform a shotgun proteomic comparison using the iTRAQ technique (see chapter 5) comparing the proteomes of *A. butzleri* ED-1 harvested from the anode of a half MFC, harvested from the planktonic phase of a half MFC and from an aerobic planktonic culture. This would show what proteins were up-regulated at the anode thus showing what proteins were potentially important in extracellular electron transfer (and hence electrogenesis) and anodic biofilm formation by *A. butzleri* ED-1. It would also show whether or not electron transporters and adhesins were up regulated at the anode and thus give some indication to how distinct the electrogenic mechanisms of *A. butzleri* ED-1 are from Geobacter *sulfurreducens* and *Shewanella oneidensis* MR-1.

1.13.2 Study and comparison of *A. butzleri* ED-1 and *Arcobacter* L biofilm formation

The study of biofilm formation has not been studied in any great detail and fluorescent imaging of *Arcobacter spp*. has not been performed. In the context of MFC biofilm formation by *A. butzleri* ED-1 is important for two reasons.

Firstly *Geobacter sulfurrreducens* and *Shewanella* oneidensis have been found to form thick conductive biofilms of 50 μm thick, which is thought to be an important part of electrogenesis by these two species (Franks *et al*., 2008). As previously discussed this is thought to be due the production of nanowires and an electrically conductive extracellular matrix which facilitate electron transfer over long distance and these thick biofilms are responsible for the high power levels generated by these bacteria (see section 1.6). Therefore imaging anodic biofilm formation over time in *A. butzleri* ED-1 would provide information as to whether *A. butzleri* ED-1 was performing electrogenesis by similar mechanisms to *Geobacter sulfurreducens* with the assumption being that if it was then it would form a thick anodic biofilm. If the electrogenic mechanisms of *A. butzleri* ED-1 were different then a thinner biofilm would be expected. Secondly it was important to compare the biofilm forming abilities of *A. butzleri* ED-1 and *Arcobacter* L. *A. butzleri* ED-1 was isolated from the anode and thought to be the more electrogenic of the two species therefore comparing biofilm formation by the two species would show whether or not the difference in electrogenesis between the two species was due to or partly due to

Arcobacter L being less able to form and maintain and anodic biofilm *than A. butzleri* ED-1.

In a more general context the study of biofilm formation by *A. butzleri* ED-1 and *Arcobacter* L was important as it prompted the development of fluorescent protein expressing vectors and methods of introducing exogenous DNA into *Arcobacter spp*. Therefore developing fluorescent vectors for studying anodic biofilm in *Arcobacter spp*. would provide useful genetic tools that could be used in a variety of projects.

Therefore the aim of studying anodic biofilm formation by *A. butzleri* ED-1 and *Arcobacter* L was to see whether they formed a thick anodic biofilm or not and thus provide an indication as to how novel the electrogenic mechanisms of ED-1 were and to see whether or not a difference in ability to form and maintain an anodic biofilm contributed to the difference in electrogenic ability observed between *A. butzleri* ED-1 and *Arcobacter* L.

As such the overall aims of the project were to use a combination of proteomic analysis and the study of biofilm formation through fluorescent imaging to investigate the electrogenic mechanisms of *A. butzleri* ED-1 by seeing how distinct they are from the previously characterised mechanisms of *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 and to determine what proteins are potentially involved in electrogenesis by *A. butzleri* ED-1. The secondary aim was to compare biofilm formation between *A. butzleri* ED-1 and *Arcobacter* L to see if the observed

differences in electrogenesis were due to their ability to form and maintain anodic biofilms.

However before either of these areas could be investigated it was important to construct a defined minimal medium for the cultivation of *A. butzleri* ED-1 and *Arcobacter* L. This could also be used to validate metabolic reconstruction information about *A. butzleri* ED-1 (Ler 2009). Therefore the first aim of the project was to construct a defined minimal medium for the growth of *A. butzleri* ED-1 and validate the metabolic reconstruction in the process.

The construction of the minimal medium is discussed in chapter 3, the study of biofilm formation in chapter 4, the iTRAQ proteomic analysis in chapter 5 and the construction of deletion mutants based on the proteomic analysis in chapter 6. Materials and methods used throughout are discussed in chapter 2.

Chapter 2

Materials and Methods

2.1 Culturing of *Arcobacter spp*.

Arcobacter spp. were cultured in 10 mL of Vandamme medium (Vandamme *et al*., 1991) which consisted of 10 g/L special peptone (Oxoid LP0072), 5 g/L lab lemco powder (Oxoid L29), 5 g/L yeast extract (Oxoid L21), 5 g/L NaCl, 2 g/L L-glutamic acid, 2g/L sodium succinate and 1 g/L $MgCl_2$ (Vandamme *et al.,* 1991). This was done in 25 mL glass universal bottle. The cultures were incubated at 30 °C under microaerobic conditions; 5% O_2 (v/v), 10% CO_2 (v/v) and 85% N_2 (v/v) (BOC) for 24-48 h, without shaking (Vandamme *et al*., 1991). The microaerobic atmosphere was created in an anaerobic jar. Unless otherwise stated this method was used to prepare the inoculum for all future experiments using *Arcobacter spp.* The bacteria were grown on solid agar plates, which were prepared as above with 15g/L of agar added.

When *Arcobacter* needed to be grown on selective medium, Houf blood agar was used (Houf *et al*., 2001). Houf blood agar consisted of Vandamme medium with 50 mL/L defibrillated horse blood and the following selective agents 100 μg/L 5-flurouracil, 16μg/L cefoperazone, 32 μg/L novobiocin and 64 μg/L trimethoprim (Houf *et al*., 2001). These were incubated as described above.

For MFC work, imaging work and growth experiments *Arcobacter* were cultured in *Arcobacter* minimal medium (AMM). AMM consisted of 3 g/L $(NH_4)_2SO_4$, 4 g/L KH_2PO_4, 0.4 g/L Na_2CO_3, 0.05 g/L NaCl, 0.01 g/L niacin, 0.24 g/L $MgSO_47H_2O$ and

0.012 g/L $FeSO_4 7H_2O$ with a carbon source added at a concentration of 30 mM unless otherwise stated. The medium was used at a pH 6.5 unless otherwise stated.

2.1.1 Viable counts of *Arcobacter spp.*

Viable counts were required for a number of different experiments. Viable counts were performed by making serial dilutions in the range of 10^{-1}-10^{-7} in 1*Phosphate buffered saline (PBS). 5 μL of each dilution was plated in duplicate onto Vandamme agar, Houf blood agar or Vandamme agar with 50 μg/mL kanamycin depending on the experiment. These plates were incubated as described in section 2.1

Viable counts of *E. coli* were performed in the same fashion only onto LB or MacConkey agar, peptone 20 g/L, lactose 10 g/L, bile salts 5 g/L, NaCl 5 g/L, neuteral red 0.075 g/L, agar 12 g/L (Oxoid CM0007) and incubated at 37 °C for 24 h.

2.2 The culture of *Arcobacter spp*. in half MFCs

Half MFCs were set up using a 250 mL Duran bottle and a strip of carbon cloth (supplied by Dr. Viatcheslav Fedorovich) to serve as a working electrode and a Ag/AgCl based reference electrode (Radiometer-analytical REF201). Half MFC set up is shown in Figure 2.1.

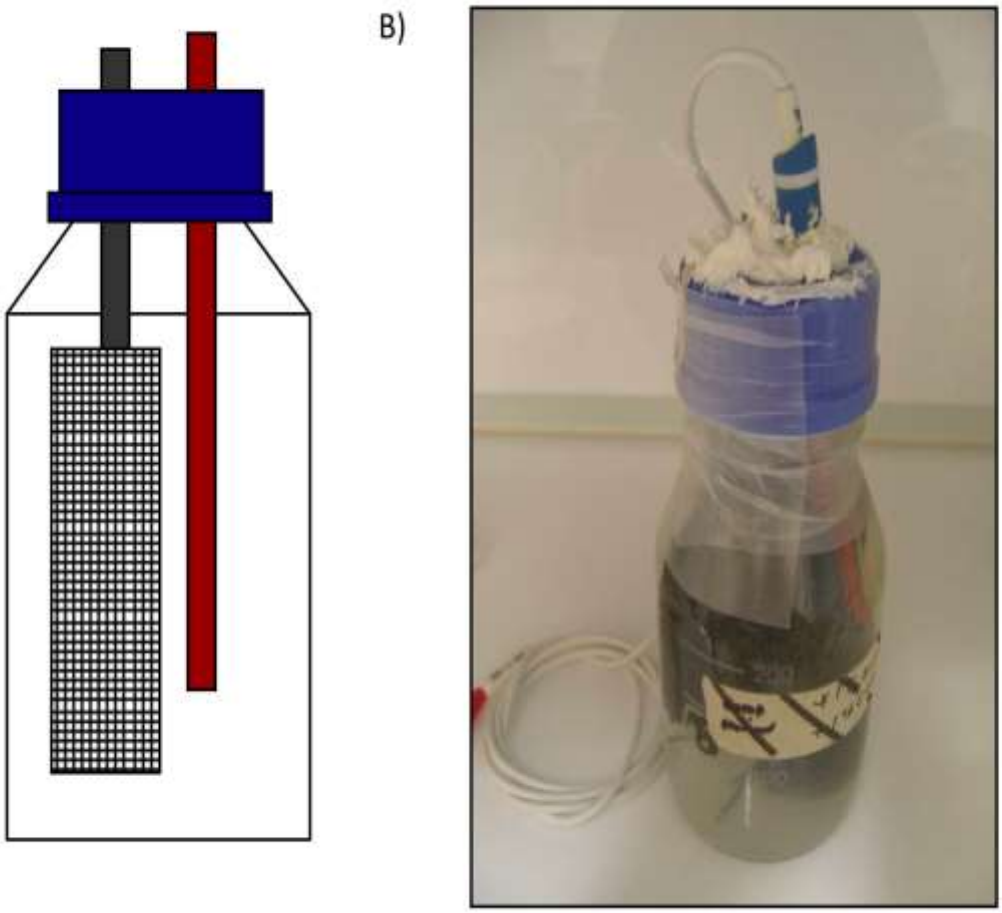


Figure 2.1, adapted from Willmoth 2010, shows a schematic diagram (A) and photograph (B) of a half MFC set up typically used. The set up consists of a working electrode made of carbon cloth and a Ag/AgCl reference electrode (radiometer-analytic REF201). Half-cells filled with AMM typically using 30mM acetate as the sole carbon source and inoculated with 4% (v/v) of either *A. butzleri* ED-1 or *Arcobacter* L incubated for 24-48 h as described in section 2.1. Half cells then tightly sealed with Parafilm and silicone sealant and then incubated at either room temperature or at 30 °C.

The half MFCs were filled with AMM and inoculated with a 4% (v/v) inoculant of *Arcobacter* and with 30 mM acetate typically serving as the sole carbon source. The half- were then sealed tightly (as shown above) and incubated at either room temperature or 30 °C.

Redox potentials were monitored by use of a voltmeter and samples of culture were taken by inserting a needle and syringe through the rubber seal in the half-cell lid.

2.2.1 Growth of *A. butzleri* ED-1 for iTRAQ analysis

A. butzleri ED-1 was cultured either aerobically or anaerobically for iTRAQ analysis. Anaerobic culture were grown in a half MFC as described above, although they were cultured under an anaerobic atmosphere of 10% CO_2 (v/v), 10% H_2 (v/v) and 80% N_2 (v/v) (BOC) at 30 °C in an MINIMAX anaerobic workstation (Don Whitley scientific). The cells were cultured for 300 h and fed every 24 h after the first 96 h via removing 20 mL of culture and replacing with 20 mL of fresh minimal medium. Aerobic cultures were grown for 72 h in AMM with 30 mM acetate serving as the sole carbon source. Cultures were inoculated with2% v/v of a pre-cultured *A. butzleri* ED-1 culture (see section 2.1) and incubated at 30 °C with 100 rpm shaking for 72 h.

Cells were harvested for iTRAQ from the planktonic phase by centrifugation at 6,000 rpm (rotor 19776, Sigma) for 20 min. in a Sigma 3-16KL centrifuge (Sigma) at 4 °C. Cells on the anode were liberated by immersion and vigorous washing in 100 mL of 1 *PBS and then centrifuged as per the planktonic cells.

2.2.1.2 Growth of *A. butzleri* ED-1- *E. coli* binary culture

The binary culture experiments were performed in a 3 anode half MFC in a volume of 500 mL of AMM with 10 mM glucose serving as the carbon source and adjusted to pH 6.5. The culture was inoculated with 1% v/v *A. butzleri* ED-1 and 1% v/v *E. coli*. The culture was seeded in batched mode for 24 h, incubated at room temperature, before being switched to continuous feeding where the culture was supplied with AMM with 10 mM glucose at a rate of 0.1 mL/min.

2.2.1.3 Growth of *A. butzleri* ED-1 using Fe^{3+} or Mn^{4+} as a terminal electron acceptor

Growth with insoluble Fe^{3+} and Mn^{4+} was performed under anaerobic conditions as described in section 2.2.1. 100 mL of AMM with 30 mM acetate serving as the sole carbon source was inoculated with 2% v/v *A. butzleri* ED-1 pre cultured in Vandamme media (see section 2.1). Insoluble $Fe3^{+}$ was supplied in the form of FeOOH and insoluble Mn^{4+} was supplied in the form of MnO_2. Soluble Fe^{3+} was supplied in the form of 10 mM ferric citrate. A positive control using 10 mM potassium nitrate as a TEA was set-up as was a negative control lacking a TEA. The cultures were incubated for a period of 40 days for 30ºC with A_{600} being measured every 7 days.

2.2.1.4 Growth of *A. butzleri* ED-1 and *E. coli* to investigate redox indicators as a screen for electrogenesis

The assays of redox indicators were performed under anaerobic conditions as described in section 2.2.1. *A. butzleri* ED-1 were cultured on Vandamme agar (see section 2.1) with 1 mM of redox indicator or 10 mM of Ferric citrate or Ferric oxyhydroxide added to the agar. *E. coli* were incubated on LB agar with redox indicator added in order to serve as a negative control. The cultures were incubated under anaerobic conditions at 30 °C for 48 h.

2.2.2. Growth of *A. butzleri* ED-1 in 96 well microtitre plates

Growth experiments were performed using AMM with various components added or subtracted depending on growth conditions being investigated. Experiments were performed in 200 μL volumes with a 2% (v/v) inoculum of *A. butzleri* ED-1 (pre cultured in AMM with 30 mM acetate serving as the sole carbon source incubated at 30 ºC under microaerobic conditions described in section 2.1) per well and 30 mM of acetate or another carbon source. Different conditions were set up in quadruplicate.

The plates were incubated at 30 °C for 48 h and readings of absorbance at 600 nm (A_{600}) were taken every 4 h using a micro titre plate reader (UMV 340 Asys scientific laboratory supplies).

2.3 Preparation of cultures for imaging

2.3.1 Imaging of *A. butzleri* ED-1 and *Arcobacter* L on microscope slides

Arcobacter containing pJK1 or pMK1 and therefore expressing green fluorescent protein (GFP) or cyan fluorescent protein (CFP) respectively were cultured as described in section 2.1 with 20 μg/mL kanamycin. Following growth the cells were spun down at 6,000rpm using rotor 19776 (Sigma) using a Sigma 3-16KL centrifuge (Sigma) ,for 20 min. and the resulting pellet re-suspended in 1 mL of 1*PBS (pH7). 10 μL of this suspension was dropped onto a microscope slide coated with a 1 in 10 dilution of poly-L-lysine (Sigma-Aldrich, P8920). The cells were left at room temperature for approximately 1 h in order to immobilise them.

. The cells were then imaged using a DeltaVision RT microscope (Applied Precision) with appropriate filters (395nm-405nm for GFP and 425nm-475nm for CFP). Images of the cells were captured using a Cascade2_1K camera (Applied Precision).

2.3.2 Preparation and imaging of *Arcobacter spp*. biofilms grown on glass coverslips

Arcobacter spp. biofilms on glass coverslips were prepared in sterilised 300 mL volume tip boxes. 25 mm by 25 mm glass coverslips were ethanol sterilised and placed in custom made metal racks. The coverslips were submerged in 200 mL of AMM with 30 mM acetate serving as the sole carbon source and supplemented with 50 μg/mL kanamycin added. They were inoculated with a 2% (v/v) inoculum of GFP tagged *A. butzleri* ED-1, GFP tagged *Arcobacter* L or a 1% (v/v) inoculum of GFP tagged *A. butzleri* ED-1 and a 1% (v/v) CFP tagged *Arcobacter* L for co-culture experiments. These cultures were prepared as described in section 2.1 with 50 μg/mL kanamycin added. The cultures were incubated statically at 30 °C. Every 24 h the medium was decanted off and replaced with 200 mL of fresh medium and antibiotics.

The biofilms were imaged by removing a glass coverslip every 24 h with ethanol-sterilised tweezers. The glass coverslip was placed on a glass side and excess liquid removed by blotting. The biofilms were imaged as described in section 2.3.1.

2.3.3 Preparation and imaging of *Arcobacter spp*. biofilms formed on a carbon anode

To prepare anodic biofilms for imaging, 3- anode versions (using carbon paper) of the half MFCs described in section 2.2 were set up with the addition of 50 μg/mL kanamycin and GFP tagged *A. butzleri* ED-1 or *Arcobacter* L as opposed to wild type. The half-cells were incubated statically at 30 °C.

A small section of anode (approximately 0.5 cm-0.5 cm) was cut every 24 h using ethanol sterilised scissors and tweezers. This was then washed by immersion in 1* PBS and placed upon a poly-L-lysine coated coverslip. Excess liquid was removed by blotting. A coverslip was affixed over the anode with vacuum grease smeared round the edge.

The sections of anode were imaged as described in section 2.3.1.

2.3.4 Preparation and imaging of samples by scanning electron microscopy (SEM)

Samples of anode were prepared for SEM analysis by cutting an approximately 1 cm^2 of anode tissue and fixing the sample with a 3 % v/v glutaraldehyde solution. The samples were washed with 1 * PBS, 0.5* PBS and deionised water before being dehydrated with 3 changes of 100% ethanol. The samples were then dried using a

critical point dryer. Samples of aerobic planktonic culture were prepared for imaging by harvesting as described in section 2.2.1 and re-suspending the pellet in 200 μL 1* PBS with an equal volume of 3 % v/v glutaraldehyde solution. 20 μL of this solution was then applied to a 25 mm by 25 mm poly-l-lysine coated coverslip (Sigma-Aldrich, P8920) before being prepared for imaging as above. Samples were images using a BIOsem scanning electron microscope using an accelerating voltage of 5 kV.

2.4 Triparental mating for the introduction of DNA into *Arcobacter spp*.

Wild type *Arcobacter* were cultured as described in section 2.1 and *E. coli* containing the helper plasmid pRK600 (Finan *et al*., 1986) was cultured in 5mL of LB with 12.5 μg/mL chloramphenicol added at 37 °C with shaking at 200 rpm overnight. The donor strain of *E. coli* (containing pJK1 or pMK1) was grown in an identical fashion with 50 μg/mL of kanamycin serving as the selective agent. Cultures were incubated in 5 mL glass universal bottles.

Each bacterium was subcultured into its respective medium with selective agents and incubated until they reached exponential phase. *A. butzleri* ED-1 and *Arcobacter* L were incubated for approximately 6 h and to an absorbance of 0.08-0.1 at 600 nm. *E. coli* were incubated for approximately 2-3 h reaching an absorbance of 02.-0.3 at 600 nm (A_{600}). *Arcobacter* were incubated in a 100 mL Duran bottle with 100 rpm

shaking at 30 °C and *E. coli* were incubated in 50 mL of LB in a 200 mL conical flask with 200 rpm shaking at 37 °C.

The bacteria were then harvested by centrifugation at 6,000 rpm at 4 °C for 20 min Sigma 3-16X centrifuge, (Sigma) using a 19776 rotor (Sigma). The resulting cell pellet as then re-suspended in 1 mL of medium lacking antibiotics. 40 μL of the suspension of each bacterium was then spread plated onto a Vandamme ager plates. The plates were incubated for 48 h at 30 °C under microaerobic conditions for 48 h.

The resulting lawn of cells was then scraped off and 10^{-1} and 10^{-2} dilutions were made of the suspension. 100 μL of undiluted suspension and each of the dilutions were plated onto Vandamme agar containing 5 μg/mL trimethoprim, 100 μg/mL ampicillin, 12.5 μg/mL chloramphenicol and 50 μg/mL kanamycin to select for recombinant *Arcobacter.* These plates were then incubated as previously described.

2. 5 The construction of pMK1

The plasmid pMK1 was constructed by replacing the GFP cassette of pJK1 with the CFP cassette from pWM1009 (Miller *et al*., 2000). Several different techniques were used for this, detailed in sections 2.5.1-2.5.4.

2.5.1 Restriction digests

The GFP cassette was excised from pJK and the CFP cassette was excised from pWM1007 by digestion with *Nco*I (NEB Ref R3193S) and *Bst*BI (NEB R0519S). Digestions were performed in 30 μL total volume with 20 μL of DNA, 3 μL of 10* NEB buffer 4 (NEB B7004S), 3μL of 10* BSA, 1 μL of *Nco*I, 1 μL of *Bst*B1 and 2 μL of sterile distilled water.

The above reaction mix was incubated at 37 °C for 3 h and then heat inactivated by incubation at 65 °C for 15 min.

2.5.2 Dephosphorylation

To increase the efficiency of ligation the pJK1 digest was dephosphorylated by the use of Antarctic phosphatase (NEB M0289S). 1 μL of Antarctic phosphatase and 3 μL of 10* Antarctic phosphatase buffer was added to the pJK1 digest before heat inactivation and incubated for 15 min. at 37 °C. Heat inactivation was performed by incubation at 65 °C for 15 min.

2.5.3 Gel purification

Following digestion the total pJK1 digest and total pWM1009 digest were subject to agarose gel electrophoresis. The samples were run out on a 1% (w/v) agarose gel made with 1*TBE buffer (diluted from a stock solution of 5*TBE made with 54 g/L Tris Base, 27.5 g/L of Boric acid and 2.92 g/L of EDTA). The gel was run at 120 V for 1 h.

Following electrophoresis the band containing the CFP cassette from the pWM1009 digest and the band containing the vector backbone from the pJK1 digest were purified by the use of a Promega SV Wizard gel purification kit. (Promega A9281).

2.5.4 Ligation

Following gel purification the CFP cassette was ligated into the vector backbone of pJK1. 5 μL of vector backbone was mixed with 15 μL of CFP cassette and

performed in 2 μL of T4 DNA ligase buffer with 1 μL of T4 DNA ligase (NEB M0202S). The ligation reaction was incubated overnight at room temperature.

2.6 Preparation and transformation of competent *E. coli*

2.6.1 Preparation of competent *E. coli* cells

Competent *E. coli* DH5α were created using an in lab protocol based upon Sambrook (Green *et al*., 2012). Competent *E. coli* was prepared from a 5 mL overnight culture grown in 5 mL of LB broth (in a glass Universal bottle) incubated at 37 °C overnight with 200 rpm shaking. A 1:100 dilution of this culture was made in 50 mL of LB in a 250 mL conical flask and incubated until the cells reached exponential phase (i.e. an A_{600} of 0.3-0.5), which takes approximately 2.5- 3 h. The cells were centrifuged at 6,000 rpm in a centrifuge set a 4°C for 20 min. (Sigma 3-16X centrifuge, Sigma) using rotor 19776 (Sigma). The cell pellet was resuspended in 3mL of ice cold 0.1M $CaCl_2$ and incubated at 4 °C overnight. The cell suspension was centrifuged at previously described, resuspended in 1 mL of ice-cold 0.1 M $CaCl_2$-20% (v/v) glycerol and divided into 50 μL aliquots before storage at -80 °C.

2.6.2 Transformation of chemically competent DH5α cells

Chemically competent *E. coli* created by the method given in 2.6.1 was transformed with 1μL of super coiled plasmid DNA or 10μL of ligation reaction mix by a protocol based on Sambrook (Green *et al*., 2012). A 50 μL volume of cells mixed with DNA was incubated on ice for 1h before heat shocking at 42 °C for 1 min. The cells were returned to ice and 950 μL of SOC medium prepared to the following

recipe was added to the reaction mix: 20 g/L peptone (Oxoid LP0037), 5 g/L yeast extract (Oxoid L21), 2 mL of 5M NaCl, 10 mL of 1M $MgCl_2$, 10 mL of 1M $MgSO_4$ and 20 mL of 1M glucose. The cells were recovered for 1 h at 37 °C. This recovery mixture was then spun down in a bench top centrifuge at 6,500 rpm for 5 min and all but a 100 μL of supernatant was removed. The pellet was then resuspended in the remaining 100 μL and plated onto LB agar containing appropriate antibiotics.

2.7 plasmid purification

Plasmid purification was required at a number of stages during the project. In order to purify plasmids a 5 mL overnight of *E. coli* was prepared with appropriate antibiotics under conditions discussed in section 2.6.1. These plasmids were then purified by use of a Qiagen miniprep plasmid purification kit (Qiagen 12123).

2.8 Analytical gel electrophoresis

The size of DNA fragments was analysed by agarose gel electrophoresis. This was performed as described in section 2.5.3 but was performed with only 5 μL of DNA and either 1 kb (NEB N3232S) or 100 bp DNA ladders (NEB N3231S).

The gels were imaged using an Epichemi II darkroom (UVP laboratory products) and Labworks 04 software.

2.9 Sanger sequencing

Sanger sequencing was performed using 5 μL of DNA and 1 μL of a working concentration of the appropriate primer and was performed by Genepool service and the University of Edinburgh (http://genepool.bio.ed.ac.uk/ 2012 Sanger *et al*., 1977). The resultant sequences were analysed using 4 peaks software (http://4peaks.en.softonic.com/mac 2012) and BLAST software at NCBI (http://www.ncbi.nlm.nih.gov/ 2012).

2.10 Attempted construction of gentamycin resistance (Gm^R) based construct for the creation of flagella deletion mutants by fusion PCR

Crossover PCR was used to attempt to create a Gm^R based cassette for the construction of flagella deletion mutant cassettes. *A. butzleri* ED-1 genomic DNA was used as the template DNA for flagella flanking sequence cassettes and the plasmid pBBR1MCS-5 (Kovach *et al.*, 1995) was used as a template for generating a Gm^R cassette. The primers used during PCR are given in Table 2.1.

Primer number	Primer name	Primer sequence (written 5' to 3')
P1	Upstream forward	ATATATCCATGG**GCCGATTTTATATATAGCTGCTGG**
P2	Upstream reverse	CGATCGATCGATCGATCGATCGAT**GCAGTTCAACAAAACGTTCTAAA**
P3	Gm^R forward	ATCGATCGATCGATCGATCGATCG**ATGTTACGCAGCAGCAACGATGT**
P4	Gm^R reverse	TTAGCCGTTACGCGTTACCGG**TTAGGTGGCGGTACTTGGGTCGA**
P5	Downstream forward	CCGGTAACGCGTAACGGCTAA**CCGCTGCTTCTTGAGCTGT**
P6	Downstream reverse	TTTTTTTTCGAA**CCAACCACACCATGCTCTACCAC**

Table 2.1 shows the 5' to 3' sequence of the primers used for crossover PCR. Restriction sites are underlined; regions of homology to template DNA are show in **bold**. Reverse primers are in the reverse complement orientation. Restriction sites are underlined *Nco*I in P1 (CCATGG) and *Bst*BI in P6 (TTCGAA)

The first round of PCR was performed in order to amplify individual fragments. It was performed using Phusion polymerase from NEB (NEB M0530S). The reagents used and the amounts are given in Table 2. 2.

Reagent	Amount (μL)
Template DNA	1
dNTPS	1
Forward Primer (P1, P3 or P5)	2.5
Reverse Primer (P2,P4 or P	2.5
Phusion polymerase	0.5
10* GC buffer	10
DMSO (for reactions with P1 and P2)	1.5
Nuclease free water	Up to 50

Table 2.2 gives the amounts in μL of reagents used for the amplification of individual fragments for crossover PCR. Phusion polymerase, buffers and DMSO supplied by NEB. dNTPs supplied by Invitrogen (N8080261).

The PCR reactions were performed under the conditions given in Table 2.3. PCR reactions were performed using MBS 0.2G PCR machines (Thermofischer Scientific).

Stage	Temperature (°C)	Time (s)
1	98	30
2 (*30 cycles)	a) 98 b) 55.3 c) 72	a) 10 b) 30 c) 20
3	72	600

Table 2.3 gives the stages of PCR, temperatures and stages they were performed at. Stage one is the initial denaturation step. Stage two consists of 30 cycles of denaturation (a) annealing (b) and extension (c). Stage 3 is the final extension step.

2.10.1 The second round of PCR: Creation of fused products

Following amplification of the individual fragments they were joined together in order to create a Gm^R based cassette for the creation of deletion mutants. The individual fragments were first ligated together using T4 DNA ligase with the following reagent mix: 10 μL of each DNA fragment, 4 μL of 10* DNA ligase buffer, 1 μL of T4 DNA ligase, 5 μL of distilled sterile water. The ligation mix was incubated at room temperature overnight. The resulting ligation mix was then used as a template for PCR.

The sample was subject to a second round of PCR to create a 2.4 kb construct; the PCR conditions were identical to those given in table 2.3 was maintained for 35 cycles. The reagents and quantities used are given in the Table 2.4.

Reagent	Quantity (μL)
Ligation mix (template)	3
DMSO	1.5
Primer P1 (forward primer)	2.5
Primer P6 (reverse primer)	2.5
dNTPs	1
10* GC buffer	10
Phusion polymerase	0.5
Nuclease free water	Up to 50

Table 2.4 gives the reagents and volumes used to perform a crossover PCR to create the final deletion mutant cassette. DMSO, Phusion polymerase and buffers were supplied by NEB and dNTPs were supplied by Invitrogen.

For both stages the presence of PCR product was confirmed by agarose gel electrophoresis as described in section 2.8.

2.11 Attempted construction of a kanamycin resistance (Km^R) based cassette for the creation of flagella deletion mutants

An alternate strategy for the creation of flagella deletion mutants was devised using different regions of flanking sequences and the Km^R cassette from pJK1. The reagents used were identical to those in Table 2.2. The primers used for this are given in Table 2.5.

Primer name	Sequence	Tm (°C)	Restriction site	Product size
P1	AACTGGAG**AAATTTGAAGCAAGAGAAAGCC**	62.388	*Xho*I (CTGGAG)	709bp
P2	TTTTTTCCATGG**GCCAAGCTAATGCAGTTCAACAA**	66.79	*Nco*I(CCATGG)	709bp
P3	ATATATCCATGG**GAAGTGCGTAAGAACATAGAAAGGC**	64.8	*Nco*I (CCATGG)	1.2kb
P4	TTTTTTCCATGG**ATAGGCAGCGCGCTTATCA**	65.65	*Nco*I(CCATGG)	1.2kb
P5	AAAAAACCATGG**CTTGAGCTGTTAAAGATGATACG**	59.43	*Nco*I (CCATGG)	813bp
P6	TTTCTAGA**GCTAAACAGGCCCAAATTAAT**	60.69	*Xba*I (TCTAGA)	813bp

Table 2.5 gives the primer name, sequence (regions of homology to template DNA shown in **bold**), melting temperature (Tm), restriction sites in the primer (underlined) and size of product the primer makes. Restrictions sites are underlined *Xho*I (CTGGAG), *Nco*I (*CCATGG*), *Xba*I (TCTAGA)

The reaction conditions for the PCR conditions are given in Table 2.6.

Stage	Temperature (°C)	Time (seconds)
1	98	30
2 (*30 cycles)	a) 98 b) 50 c) 72	a) 10 b) 30 c) 20
3	72	600

Table 2.6 gives the stages of PCR, temperatures and stages they were performed at. Stage one is the initial denaturation step. Stage two consists of 30 cycles of denaturation (a) annealing (b) and extension (c). Stage 3 is the final extension step.

Following PCR fragments were analysed using gel electrophoresis as described in section 2.8 and purified as described in section 2.5.3.

2.11.1 Restriction digests and ligation

The purified fragments were digested as described in 2.5.1 although using *Xba*I (NEB R0145S) and *Xho*I (NEB R0146S) as opposed to *Bst*B1. The fragments were then ligated together as described in 2.5.3 and then ligated into pBBR1MCS-5 digested with *Xba*I and *Xho*I and dephosphorylated as previously described (see section 2.5.2).

The resulting ligation mixture was then transformed into chemically competent *E. coli* DH5α as previously described.

Chapter 3

Creation of a defined minimal medium for the growth of *A. butzleri* ED-1 and *Arcobacter* L

3.1 Introduction

Arcobacter spp. have been traditionally cultured on rich media such as Vandamme medium (Vandamme *et al.,* 1991), Houf blood medium (Houf *et al.,* 2001) and Brain heart infusion medium (Ho *et al.,* 2008). However rich media were not suitable for further study of the growth of *Arcobacter spp.* due to their poorly defined composition of complex substrates and were unsuitable for imaging due to the presence of molecules such as tryptophan and tyrosine which produce high levels of background fluorescence, which would make the planned florescent imaging (discussed in chapter 4) unfeasible.

The *Arcobacter* were originally isolated from a MFC fed with an acetate minimal medium of pH 5.8 (Fedorovitch *et al*. 2009). This minimal medium was unsuitable for the growth of *A. butzleri* ED-1 and *Acobacter* L as it produced low growth even when adjusted to a more suitable pH of 6.5 and frequently produced highly variable redox potentials (data not shown). The variable redox potential was possibly due to high levels of nitrate in the local tap water, which *Arcobacter* are capable of using as a terminal electron acceptor due to possessing nitrate reductases (Miller *et al*., 2007).

It was decided to devise a minimal medium for *Arcobacter* growth based on a *Campylobacter* minimal medium (CMM), taken from Roup *et al*. 1984, due to various physiological similarities between *Arcobacter spp*. and *Campylobacter spp*. These similarities include being non sacchrolytic (i.e. obtain energy from short chain

organic acids and amino acids as opposed to sugars), nitrate and nitrite reduction and a requirement of 1-10% CO_2 for growth (Kelly 2001).

3.1.2 Aims and objectives

The first aim of the study was to create an *Arcobacter* minimal medium, optimised for the growth of *A. butzleri* ED-1 and *Arcobacter* L using an existing CMM recipe (Roup *et al*., 1984). The CMM was adjusted to a pH of 6.5 as previous experiments showed that *A.butzleri* ED-1 exhibited strong growth at that pH and its rich medium also used at this pH (Vandamme *et al*., 1991). Therefore all experiments were performed at this pH unless otherwise noted.

The second objective was to investigate the range of carbon sources *A. butzleri* ED-1 was capable of growing on. Metabolic reconstruction and genomic analysis predicted that the bacteria could grow utilising acetate, lactate, fumarate, propanoate, malate and succinate (Ler 2009). Therefore it was decided to investigate the growth of *A. butzleri* ED-1 on these carbon sources as well as a variety of other carbon sources in order to validate the metabolic reconstruction and to see what types of MFC *A. butzleri* ED-1 could be used in on a larger scale.

The ability of a binary culture of *A. butzleri* ED-1 and *E. coli* to electrogenically degrade glucose was also studied. *A. butzleri* ED-1 is unable to use sugars such as

glucose and other complex molecules as its sole carbon and energy source (Miller *et al*., 2007, Ler 2009) this limits its use in large scale MFCs which typically use complex mixture of molecules as a fuel supply; examples include winery wastewater where a 1000 L MFC was used to produce hydrogen over a 100 day time period at a rate of 0.19 L/L/day (Cusick *et al*., 2011) and brewery wastewater, although performance of this device remains unreported in the literature (Logan 2010). Therefore a microbial consortium capable of dealing with a complex mix of molecules is essential for use in large scale MFC. The *A. butzleri* ED-1 *E. coli* binary culture was to be the first stage in creating a specifically designed MFC microbiological consortium for the electrogenic degradation of cellulose.

The final aim of the growth experiments was to investigate the ability of *A. butzleri* ED-1to grow using insoluble Fe^{3+} and Mn^{4+} as TEAs. *Arcobacter spp*. were found to be the dominant species in an anaerobic environment where manganese reduction was taking place (Thamdrup *et al*. 2000), however the growth of the bacteria using manganese or iron as a terminal electron acceptor had not been quantified nor had the reduction of the insoluble metal ions. Therefore the goal was to quantify the growth of *A. butzleri* ED-1 using insoluble Mn^{4+} and Fe^{3+} as terminal electron acceptors and quantify the reduction of the metal ions as well. The ability to culture *A. butzleri* ED-1 on insoluble metal ions could be useful for the study of its pathways of extracellular electron transfer and electrogenesis as the process is similar to dissimilatory metal reduction.

3.2 Results

3.2.1 The growth of *A. butzleri* ED-1 and *Arcobacter* L in *Campylobacter* minimal medium

A.butzleri ED-1 and *Arcobacter* L were grown in CMM with 30 mM acetate serving as the sole carbon. Two sets of growth experiments were performed; growth in CMM after bacteria were subcultured from an overnight culture prepared in Vandamme medium (see section 2.1) and growth in CMM following subculture from a culture of CMM in order to test whether growth was due to "carry over" nutrients from the Vandamme medium. Figure 3.1 shows the growth of the bacteria over a 72 h time period.

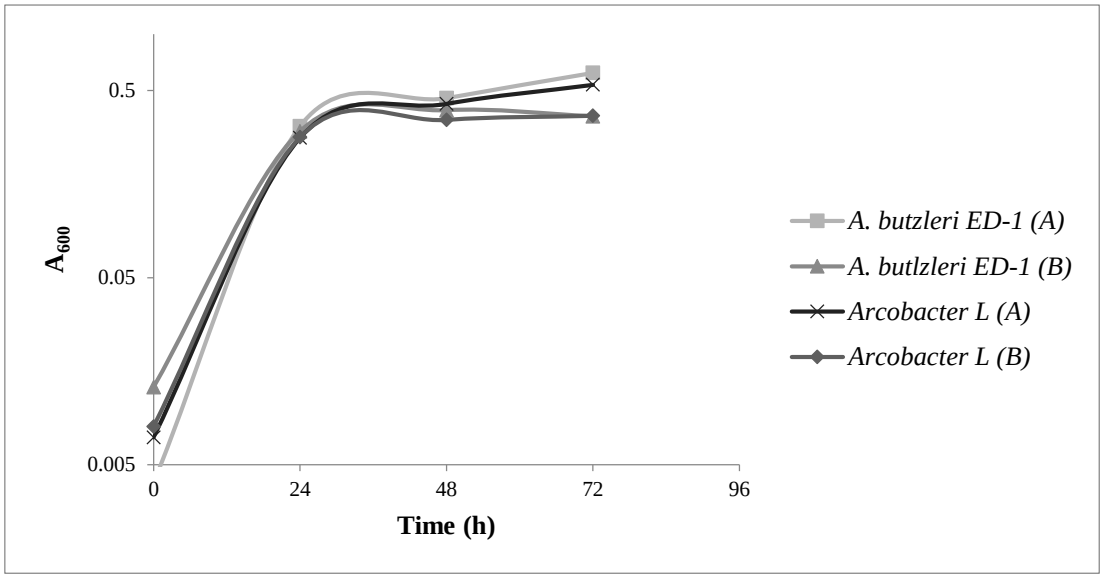
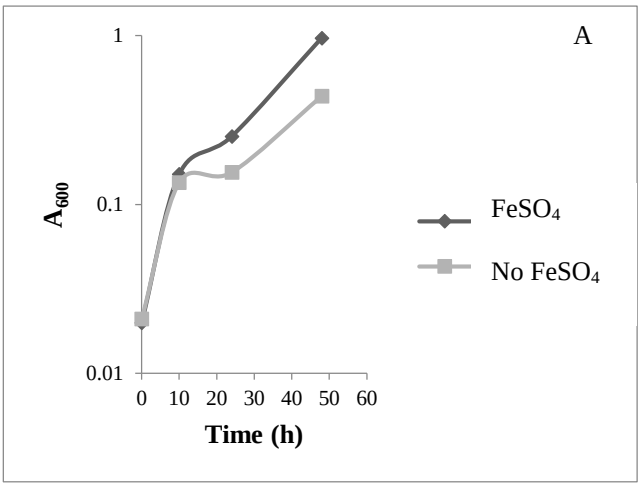


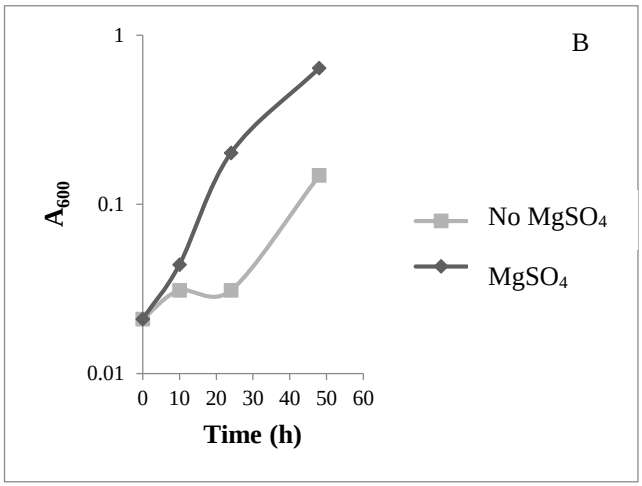
Figure 3.1 shows the growth of *A. butzleri* ED-1 and *Arcobacter* L in CMM (pH 6.5 with 30 mM acetate added as a carbon source) over a time period of 72 h. Cultures A were cultures grown from Vandamme overnight cultures and cultures B were grown by sub culturing culture A. Cultures were grown in 10 mL universal glass bottles at 30 °C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2). Final A_{600} reached was 0.621 (*A. butzleri* ED-1 (A)), 0.321 (*A. butzleri* ED-1 (B)), 0.536 (*Arcobacter* L (A)) and 0.333 (*Arcobacter* L (B)). This suggests that while carry over nutrients were not required for *A. butzleri* ED-1

A. butzleri ED-1 and *Arcobacter* L successfully grew in CMM with both sets of culture growing at identical rates between 0 and 24 h. The growth of both sets of culture B suggested that "carry- over" nutrients from the Vandamme overnight culture were not required for growth.

3.2.2 The importance of $MgSO_4$, $FeSO_4$ and $Na_2S_2O_3$ for the growth of *A. butzleri* ED-1.

To investigate whether $MgSO_4$, $FeSO_4$ or $Na_2S_2O_3$ were essential for *A. butzleri* ED-1; the bacterium was grown in two preparations of CMM lacking the desired mineral of interest and after 10 h of growth one of these cultures was supplemented with the mineral of interest; the results of this experiment are shown in Figure 3. 2.





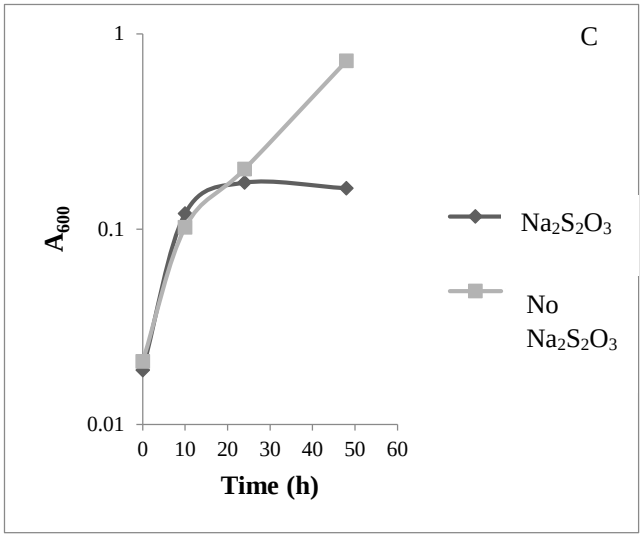


Figure 3. 2 shows the growth of *A. butzleri* ED-1 on CMM medium with and without $FeSO_4$ (A), $MgSO_4$ (B) and $Na_2S_2O_3$ (C) over a time period of 48 h. $FeSO_4$, $MgSO_4$ or $Na_2S_2O_3$ was added after 10 h of growth (indicated by red arrow). The growth experiments show that $MgSO_4$ was essential for growth as cultures lacking $MgSO_4$ were unable to grow after 10 h of growth (growth up to this point was presumably due to carry over nutrients). $FeSO_4$ was found to be useful but no required for growth as cultures lacking $FeSO_4$ showed a reduced growth. $Na_2S_2O_3$ was found to be inhibitory to the growth of *A. butzleri* ED-1

Culture A over the first 10 h grew to an A_{600} of 0.1 before the addition of $FeSO_4$ to one of the cultures. Growth than proceeded for both cultures at an identical rate until the 48 h time point where the culture with $FeSO_4$ added reached a final A_{600} of approximately 1 whereas the culture without $FeSO_4$ reached a final A_{600} of approximately 0.8. This suggested that Fe^{2+} enhanced the growth of *A. butzleri* ED-1 but was not essential.

Culture B over the first 10 h grew to an A_{600} of approximately 0. 05; after the addition of $MgSO_4$ to one of the cultures growth rapidly increased and reached an A_{600} of approximately 0.8. The culture without $MgSO_4$ added continued to grow after a plateau between 10 and 24 h and reached a final A_{600} of approximately 0.1. The purity of both cultures was verified by light microscopy. This suggested that Mg^{2+} was essential for *A. butzleri* ED-1 growth.

Culture C grew to an A_{600} of approximately 0.1 after 10 h of growth. The addition of $Na_2S_2O_3$ to the culture at the 10 h time point inhibited the growth of the bacterium, with A_{600} remaining at 0.1 until the final 48 h time point. The culture C without $Na_2S_2O_3$ added at the 10 h point continued to grow at a rate similar to that of culture A until an A_{600} of approximately 1 was reached. This suggested that $Na_2S_2O_3$ inhibited the growth of *A. butzleri* ED-1.

Therefore it was determined that Mg^{2+} was essential for growth and the presence of Fe^{2+} enhanced but was not required for growth. As $Na_2S_2O_3$ was inhibitory it was omitted from further basal medium recipes.

3. 3.4 The necessity of aspartate, glutamic acid and leucine for *A. butzleri* ED-1 growth

CMM contained the amino acids aspartate, glutamic acid and leucine (Roop *et al*., 1984). Metabolic reconstruction (Ler 2009) suggested that *A. butzleri* ED-1 did not require amino acids for growth and was capable of synthesising all 20 amino acids from biosynthetic precursors. However it was important to verify this experimentally.

To that end *A. butzleri* ED-1 was cultivated in 96 well microtitre plates in CMM lacking $Na_2S_2O_3$ with 30 mM acetate serving as the sole carbon source. These experiments were performed in quadruplicate in 96 well microtitre plates (see chapter 2, section 2.2.2). The averages of these quadruplicates were plotted as shown in Figure 3.3.

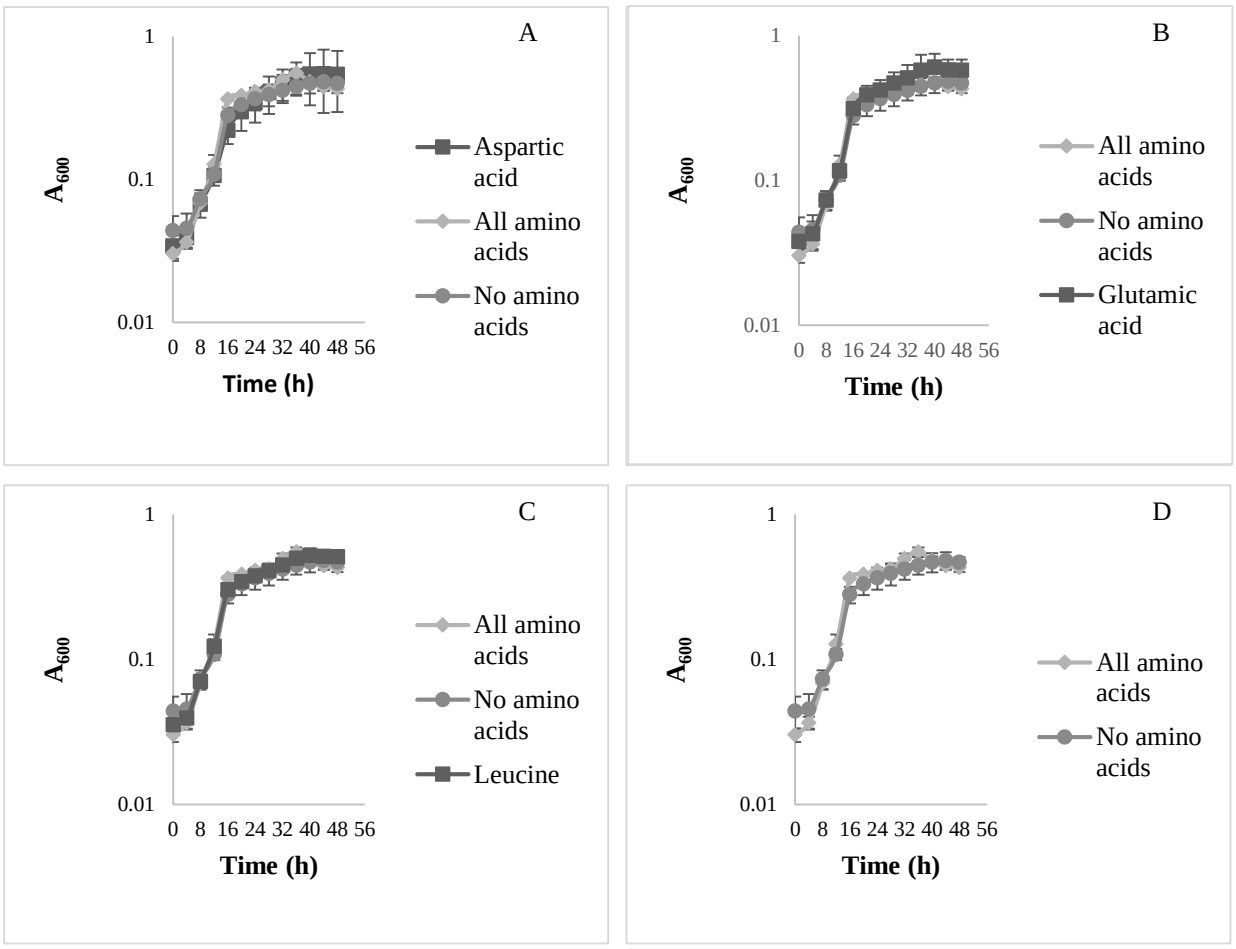


Figure 3.3 the growth of *A. butzleri* ED-1 on CMM (lacking $Na_2S_2O_3$ and with 30 mM acetate serving as a carbon source, pH 6.5) with and without asparatic acid (A), glutamatic acid (B) , leucine (C) and no amino acids (D) over 48 h with A_{600} measured every 4 h. The micro titre plates were incubated statically at 30 °C. The final A_{600} were 0.54, 0.57 and 0.51 for cultures without aspartic acid, glutamic acid and leucine respectively and 0.43 and 0.47 for cultures with all the amino acids and cultures without any amino acids respectively.

The presence of amino acids made no difference to the rate of growth with rate of growth being identical between each culture. Cultures grew most between the 4 h

and 16 h time point, after which the rate of growth slowed until finally plateauing at 32 h. Cultures with glutamic acid had a final growth yield of A_{600} 0.57 whereas cultures with all amino acids had a final A_{600} of 0.43 and cultures without any amino acids had a final A_{600} of 0.47. Based on this information it was decided to omit the amino acids from the AMM recipe as they were not deemed essential for growth.

3.2.5 Investigation to see whether AMM would support electrogenic growth

AMM was found to support the growth of *A. butzleri* ED-1 but for further use in investigating *A. butzleri* ED-1 it was unknown whether the medium would support electrogenic growth, a critical factor for using the medium to further study *A. butzleri* ED-1 within an MFC.

In order to investigate this half MFCs were set up (see chapter 2) and *A. butzleri* ED-1 was cultured in a half MFC using acetate or lactate as the sole carbon source. In addition the electrogenic abilities of *A. butzleri* ED-1 and *Arcobacter* L were compared using AMM with 30 mM acetate as the sole carbon source to confirm the claims that *A. butzleri* ED-1 was more electrogenic than *Arcobacter* L (Fedorovich *et al*., 2009). The results for these two sets of experiment are shown in Figures 3.4 and 3.5 respectively.

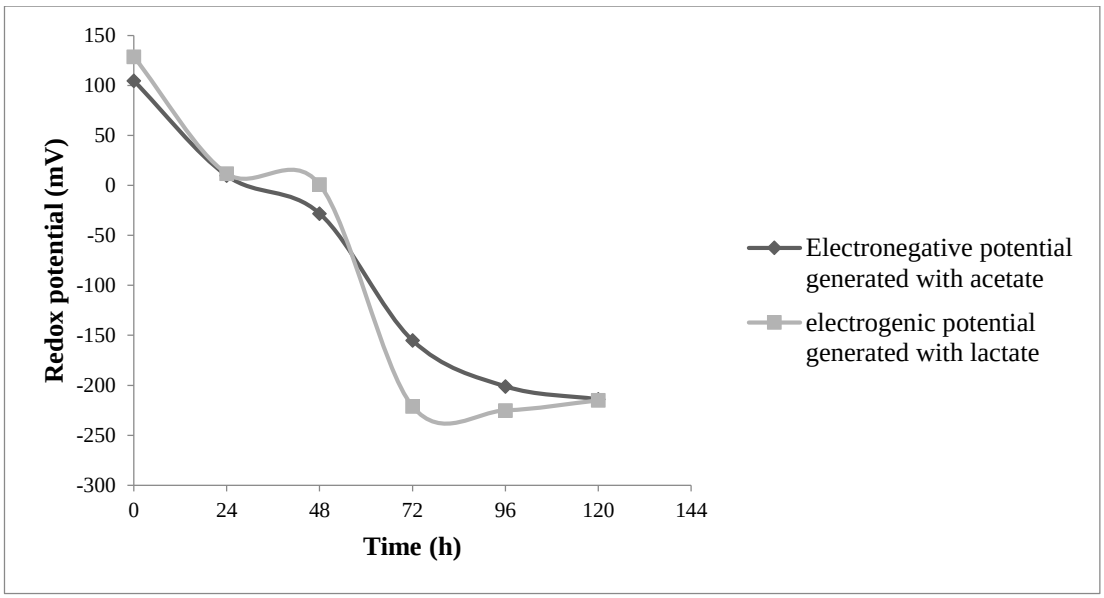


Figure 3.4 shows the redox potential *A. butzleri* ED-1 cultured in a half MFC over a 120 h time period in AMM using either 30 mM acetate or 30 mM lactate as the sole carbon source. The carbon source used has little effect on the redox potential generated by the bacteria although the redox potential of the *A. butzleri* ED-1 cultured using lactate drops more rapidly between 48 and 72 h. The carbon source used has no effect on the final, stable redox potential achieved by both cultures after 120 h, with both cultures reaching redox potentials of approximately -225 mV.

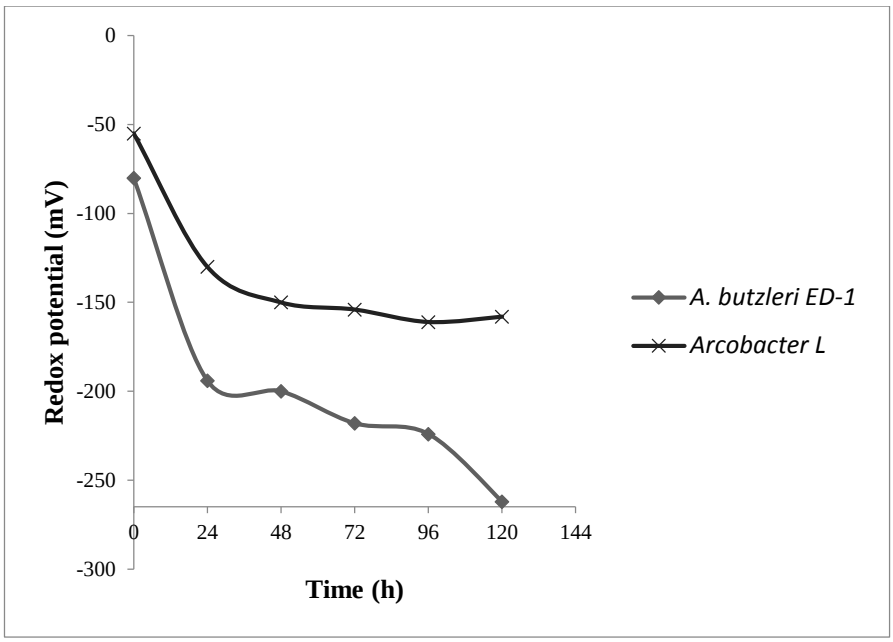


Figure 3.5 shows the redox potentials generated by *A. butzleri* ED-1 and *Arcobacter* L over a 120 h time period. The bacteria were cultured in half cells using AMM with 30 mM acetate serving as the sole carbon source. The redox potential of *A. butzleri* ED-1 reaches a much more negative (-200 mV as opposed to -70 mV) value after 24 h than *Arcobacter* L and continues to fall after 48 h and finally reaching a redox potential of -275 mV after 120 h. By contrast *Arcobacter* L reaches redox potential of -135 mV after 48 h and remains at that potential for the duration. This suggests that *A. butzleri* ED-1 was more electrogenic than *Arcobacter* L as predicted by Fedorovitch *et al*., 2009. More experiments would be required to confirm this.

A culture of *A. butzleri* ED-1 grown using acetate and a culture grown using lactate both generated a redox potential of – 225 mV after 120 h of growth in a half MFC. The culture grown using lactate as the carbon source reached a more negative redox potential than the acetate grown culture after 72 h (-250 mV as opposed to -150 mV) although after this the redox potential of the lactate grown culture rose to -225 mV whereas the acetate based culture continued to decline.

The culture of *A. butzleri* ED-1 in Figure 3.5 generated a more negative redox potential than the culture of *Arcobacter* L. During the first 24 h the redox potential of both cultures fell at same rate with the culture of *A. butzleri* ED-1 reaching a redox potential of -200 mV and the culture of *Arcobacter* L reaching a redox potential of -125 mV. After the first 24 h the redox potential of the *A. butzleri* ED-1 culture, continued to decline at a slower rate until it reached a redox potential of -260 mV after 120 h. The redox potential of the *Arcobacter* L culture declined by a small amount between 24 h and 48 h to a value of -150 mV, which remained constant throughout the duration of the experiment.

3. 2.6 Carbon source utilisation by *A. butzleri* ED-1

Genome sequencing and metabolic reconstruction (Miller *et al.,* 2007, Ler 2009) suggested that *A. butzleri* ED-1 was able to utilise a variety of organic acids as carbon sources. *A. butzleri* ED-1 has been predicted to be able to utilise acetate, lactate, propanoate, succinate and malate as a carbon source (Ler 2009). It was decided to verify this experimentally, by culturing *A. butzleri* ED-1 in AMM supplemented with different carbon sources using a 96 well microtitre plate set up (see chapter 2 section 2.2.2 for full details of set up and culture conditions). The experiments were set up in quadruplicate and the averages were plotted onto growth curves. The growth curves of *A. butzleri* ED-1 on a variety of different carbon sources is shown in Figure 3.6.

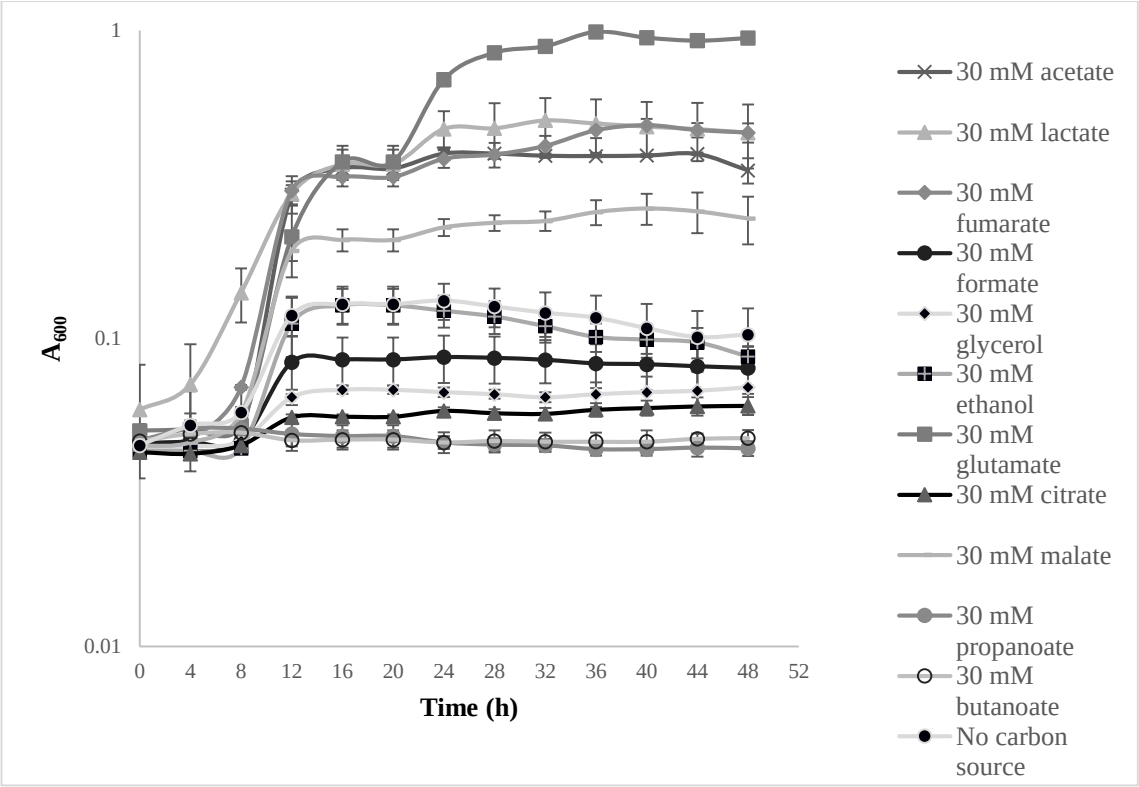


Figure 3. 6 compares the growth of *A. butzleri* ED-1 on a variety of different carbon sources to growth on no carbon source at a pH of 6.5 over a 48 h time period with A_{600} being measured every 4 h (growth curves show the mean value taken from a quadruplicate set of cultures). The final A_{600} values are 0.35 (acetate), 0.47 (lactate), 0.46 (fumarate), 0.08 (formate), 0.07 (glycerol), 0.09 (ethanol), 0.95 (glutamate), 0.06 (citrate), 0.25 (malate), 0.044 (propanoate), 0.047 (butanoate) and 0.103 (no carbon source). The small amount of growth without a carbon source is due to "carry over" nutrients from the original inoculant. Cultures containing formate, glycerol, ethanol, propanoate and butanoate exhibited less growth than cultures without a carbon source which suggests that these carbon sources are inhibitory to *A. butzleri* ED-1 growth.

Figure 3.6 showed that *A. butzleri* ED-1 exhibited strong growth on acetate, lactate fumarate and glutamate and showed weak growth on malate. When using glutamate

as a carbon source the bacterium exhibited growth for 16 h than growth plateaued for 4 h and then a second phase of growth occurred between 16 and 36 h. The purity of the glutamate cultures was confirmed by light microscopy and plating on selective and non-selective agar at the end of the incubation period (data not shown).

The cultures utilising formate, glycerol, citrate, ethanol, butanoate and propanoate did not exhibit any growth. A small amount of growth was observed when using formate and ethanol in the first 12 h of incubation, as was the same amount of growth in cultures lacking a carbon source. No growth was observed in the cultures using propanoate, butanoate and citrate as their sole carbon source.

The purity of these cultures was assessed by light microscopy and plating onto selective and non-selective agar at the end of the incubation period (data not shown).

3.2.7 The creation of an *E. coli-A. butzleri* ED-1 binary culture for the electrogenic degradation of glucose (work performed in collaboration with Chris Willmoth)

A. butzleri ED-1 lacks 6- phosphofructokinase, hexokinase, glucokinase and a glucose uptake system and so it is incapable of using glucose as a carbon source directly. However as shown in section 3.2.6 the bacterium is capable of using lactate and acetate as a carbon source. These carbon sources are the product of glucose fermentation and as such *A. butzleri* could be grown in co-culture with a bacterium capable of fermenting glucose thus producing acetate and lactate which *A. butzleri* ED-1 could then oxidise and generate electricity from. This would mean glucose was degraded and electricity generated by culturing a glucose fermenter and *A. butzleri* ED-1 together in a MFC.

It was decided to use *E. coli* B as a glucose fermenter as this bacterium is known to ferment glucose to acetate, formate and ethanol and is incapable of anaerobic growth using acetate; therefore the *E. coli* would produce organic acids by the fermentation of glucose and *A. butzleri* ED-1 could grow electrogenically using the acetate without competition from *E. coli* therefore resulting in a strongly electrogenic system. The *E. coli* B- *A. butzleri* ED-1 binary culture was intended as the first step in creating an MFC system for the electrogenic degradation of cellulose.

3.2.7.1 The dynamics of the binary culture after the first 24 h of growth (work performed by Chris Willmoth)

A binary culture of *A. butzleri* ED-1 and *E. coli* B was grown in a half-cell set up as described in Materials and Methods (section 2.2) with 10 mM glucose serving as the sole carbon source. The aim of the experiment was to see how the redox potential and cell numbers changed over 24 h of growth as shown in Figure 3.7.

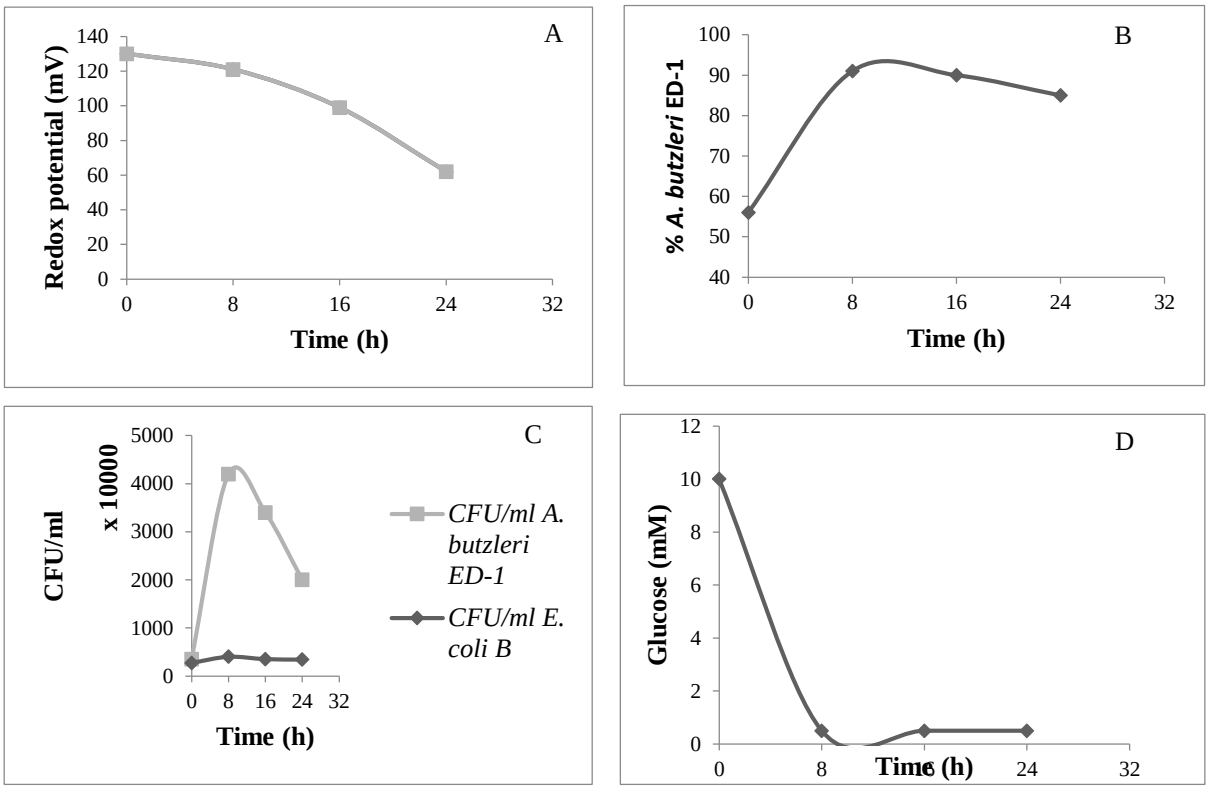


Figure 3.7 shows the redox potential (A), cell numbers (B), % of *A. butzleri* ED-1 (C) and glucose concentration (D) of a batch binary culture over 24 h with readings being taken every 8 h. Cultures were incubated at 30 ◦C. It can be seen that within 8 h only 0.5 mM of glucose remained and *A. butzleri* ED-1 had established itself as the dominant species in the binary culture, comprising 90% of the culture and although the numbers halved between 8 and 2 h they still remained the dominant species in the system. Despite the depletion of glucose *E. coli* did not increase in number by a significant amount. A fall in redox potential was observed however after 24 h the redox potential was 50 mV, which is much higher than redox potential observed for pure culture of *A. butzleri* ED-1. The results were taken from a single culture and therefore must be repeated.

It can be seen from Figure 3.7 that *A. butzleri* ED-1 rapidly proliferated within the binary culture and established itself as the dominant species within the culture after 8 h of growth and that the number of *E. coli* does not significantly increase within the 24 h time period going from 2.7 $*10^6$ to 4.0 $*10^6$ CFU/mL over 8 h and then remaining constant for the rest of the 24 h. The number of *A. butzleri* ED-1 increased to 4.2 * 10^7 CFU/mL after 8 h although this dropped to 2.0 $*10^7$ CFU/mL after 24 h of incubation.

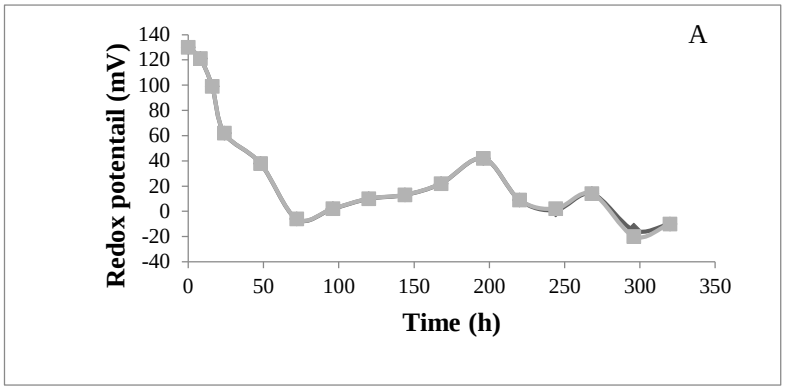
Glucose was consumed over the first 8 h with the concentration of glucose going from 10 mM to 1 mM, which correlated with the small increase in *E. coli* numbers and the increase in *A. butzleri* ED-1 numbers.

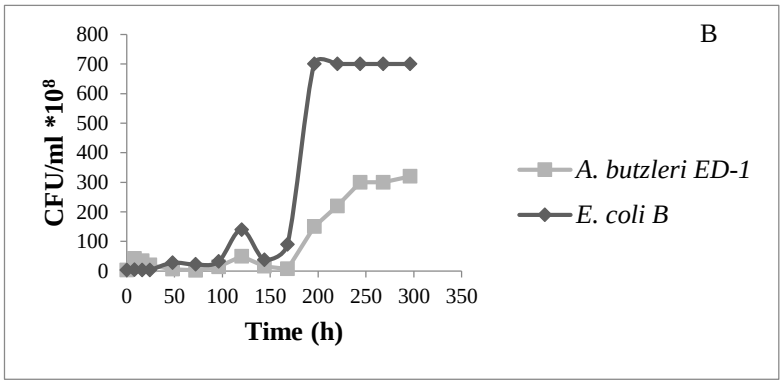
The redox potential of the culture declined from 120 mV to 80 mV over the 24 h time period, which was significantly less negative than pure *A. butzleri* ED-1 culture which typically reached redox potentials of 100 mV dependent on incubation temperature and prior deoxygenation of the basal medium.

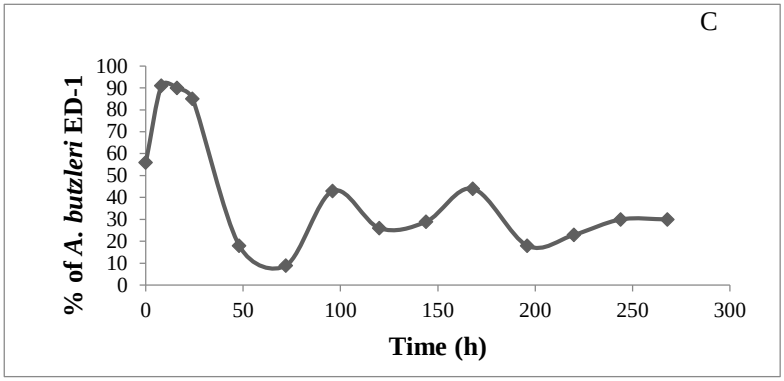
3.2. 7.2 The continuous culture of an *A. butzleri* ED-1 *E. coli* binary culture over a 300 h time period.

To further investigate the dynamics of the *A. butzleri- E. coli* binary culture under chemostat conditions i.e. with a continuous supply of nutrients. This would allow a more thorough understanding of the binary culture to be obtained as lack of nutrients would not be a limiting factor. This would show whether the system would be suitable for the electrogenic degradation of glucose.

A chemostat half-cell was set up with AMM as the basal medium with 10 mM glucose serving as the sole carbon source fed continuously into the system. The system was seeded for 24 h after which feeding started. The culture was maintained until a steady state was reached which took approximately 300 h. Figure 3.8 shows the redox potential and cell numbers obtained over the 300 h time.







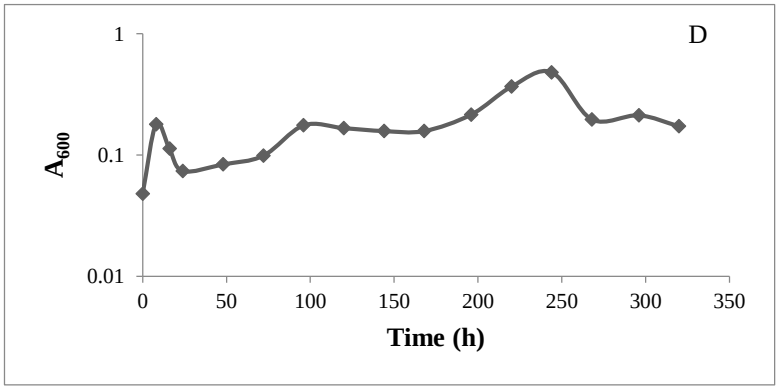


Figure 3.8 shows the redox potential (A), cell numbers (B), % *A. butzleri* ED-1 (C) and growth (D) of a binary *A. butzleri- E. coli* culture over 300 h with 10 mM glucosse serving as the sole carbon source. The culture was incubated at room temperature and readings were taken every 24 h. It can be seen that the after 300 h the culture is weakly electrogenic, at most reaching a redox potential of –20 mV. After the first 24 h (see figure 3. 12) the % of *A. butzleri* ED-1 rapidly drops off until 50 h where it comprises 10% of the population with *E. coli* starting to out compete the *A. butzleri*. After 150 h there is a large increase in both *E. coli* and *A. butzleri* numbers, with *E. coli* remaining the dominant species in the culture, with the *E. coli* entering a "steady state" after 200 h. The number of *A. butzleri* ED-1 enters a "steady state" at 250 h where it comprises 30% of the population.

During the first 24 h the cell numbers were similar to those observed for the batch fed experiment described in section 3.3.7.1 with numbers of *E. coli* increasing slightly and numbers of *A. butzleri* ED-1 rapidly increasing during the first 8 h where it established itself as the dominant bacterium within the population before declining in number after the 8 h time point, although still remaining the dominant species. Glucose was also depleted within this first 24 h with the concentration dropping to 1 mM.

When continuous feeding was initiated the number of *A. butzleri* ED-1 continued to decline until it formed only 10 % of the population within the system with the number of *E. coli* increasing within the system. After 150 h of growth there was a large spike in the number of *E. coli* within the system and while this corresponded with a spike in the number of *A. butzleri* the *E. coli* remained the dominant bacterium within the system with *A. butzleri* ED-1 comprising only 30 % of the population by the end of the experiment. The number of *E. coli* stabilised after 200 h of growth and the number of *A. butzleri* stabilised after 250 h of growth. A sample of the anodic population was taken at the end of the experiment; this showed that *E. coli* formed >90% of the anodic population (data not shown).

The redox potential of the system declined to -10 mV, which as stated previously was much less than the redox potential associated with pure culture of *A. butzleri* ED-1. The redox potential fluctuated increased to 30 mV after 200 h of growth

before declining again, eventually reaching a redox potential of -10 mV at the end of the systems run.

3.2. 8 The growth of *A. butzleri* ED-1 on metal oxides and the use of metal oxides

The growth of *A. butzleri* ED-1 using Mn^{4+} and Fe^{3+} was investigated over a time period of 40 days by incubating the bacterium with either MnO_2, FeOOH or Ferric citrate serving as a terminal electron acceptor. A positive control with 10 mM NO_3^- serving as an electron acceptor and a negative control lacking a TEA were also set up.

The results were unclear as to whether *A. butzleri* ED-1 grew on any of the TEA supplied as some growth was observed on the culture lacking TEA and in the culture containing MnO_2 data not shown).

Another experiment with FeOOH was conducted and the culture left for a month; this produced a colour change in the FeOOH precipitate from brick red to blue black (data not shown) which is indicative of Fe^{3+} reduction to Fe^{2+}. Cell counts of the resultant biofilm showed it to be a pure culture of *A. butzleri* ED-1 (data not shown)

3. 3 Discussion

3.3.1 Creation of *Arcobacter* minimal medium

Mg^{2+} was found to be essential for growth; with no further growth being observed after 10 h in the culture not supplemented with $MgSO_4$. Fe^{2+} was found to enhance growth but was not essential for growth as supplemented and non-supplemented cultures both exhibited growth after 10 h. This result was unsurprising as Mg^{2+} serves as a co factor for enzymes such as DNA polymerase, which are required for growth. Therefore Mg^{2+} was thought to be essential for *A. butzleri* ED-1 growth as it served as a co-factor for enzymes vital for cell growth. It must be noted that these results were taken from a single set of experiments and should be repeated using the microtitre plate set up (see section 3.3.3) in order to produce results in quadruplicate.

$Na_2S_2O_3$ was found to inhibit growth as the addition of $Na_2S_2O_3$ stopped at the10 h mark prevented any further growth *of A. butzleri* ED-1. *A. butzleri* ED-1 contains several genes for sulfur uptake, assimilation and biosynthesis of sulfur containing amino acids (Ler 2009, Toh *et al*., 2011) and therefore should be able to utilise $Na_2S_2O_3$. It is possible that while present in the genome these enzymes are inactive in *A. butzleri* ED-1; otherwise it is unknown why $Na_2S_2O_3$ inhibited the growth of *A. butzleri* ED-1. It must be noted that this experiment was taken from a single set of data points and therefore should be repeated using a microtitre plate set up in order to

achieve results with greater reproducibility and confirm whether or not the inhibitory effects of $Na_2S_2O_3$ on *A. butzleri* ED-1 growth were consistent.

There was no significant difference observed in growth between *A. butzleri* ED-1 grown without one or more of the amino acids and *A. butzleri* ED-1 cultivated without amino acids. This showed that the bacterium did not require any additional amino acids for growth. This was an unsurprising result as the metabolic reconstruction predicted that *A. butzleri* ED-1 was capable of synthesising all 20 amino acids (Ler 2009).

It was decided to approach the creation of AMM using a traditional approach i.e. find the recipe of an existing minimal medium, in this case the CMM described by Roup *et al*. Omission experiments were then performed to determine what components of the medium were required for growth and then use the resulting new medium to experimentally validate the metabolic reconstruction. This approach was decided upon as it is a simple approach that has yielded success with a wide range of different bacteria. For example two minimal media for *Lactobacillus plantarum* were developed by using a chemically defined medium and performing omission experiments to determine what components were essential for growth and confirm genomic reconstructions (Wegkamp *et al*., 2010). Similar approaches of omitting components from chemically defined media to determine which are essential for growth have been used for *Streptococcus thermophilus* (Letort and Julliard 2001). The approach has been used for *Campylobacter spp.* where it was used to develop a

defined chemical medium for auxotyping of *C. jejuni* (Dickgeisser and Czylwick 1985). The experiments above are the first time such an approach has been used to develop a minimal medium for the cultivation of *Arcobacter spp*. and AMM and represents a useful tool for the further study of Arcobacter *spp*. An alternative approach to the creation of AMM would have been to create a minimal medium based upon the information provided by the metabolic reconstruction. This approach has been used to create minimal media for *Staphylococcus aureus* (Becker and Palsson 2005, Lee *et al*., 2009) and for *Xylella fastidiosa* (Bhattacharyya *et al*., 2002) so there is precedent to use this approach as opposed to the traditional approach. However a traditional approach was decided upon as availability of a defined medium as a starting point meant that growth experiments could be initiated more quickly as the CMM already supported *Arcobacter spp*. growth (see section 3.2.1) and therefore the traditional approach was more suited to the time constraints of the project. However while the metabolic reconstruction was not used to create a minimal medium it was used to explain the results obtained from the growth experiments.

Therefore it was concluded that the *A. butzleri* ED-1 did not require aspartate, glutamic acid or leucine to grow and that the assumptions of the metabolic reconstruction with regards to amino acid synthesis were correct i.e. *A. butzleri* ED-1 was capable of synthesising all 20 amino acids from biosynthetic precursors. Aspartic acid, glutamic acid and leucine were omitted from further basal medium recipes.

Based on these results it was thought that Mg^{2+} and Fe^{2+} were required for *A. butzleri* ED-1 growth and $Na_2S_2O_3$ was inhibitory to the growth of *A. butzleri* ED-1 however more experiments were needed to confirm that this was reproducible across multiple cultures and could be definitively concluded. It was concluded that *A. butzleri* ED-1 did not require additional amino acids for growth. Finally it was concluded that the traditional method for creating a minimal medium and then using it to validate metabolic reconstruction data was a valid approach for the creation of an *Arcobacter* minimal medium and that this is the first time a minimal medium has been created specifically for the cultivation of *Arcobacter spp.*

3.3.2 The electrogenic growth of *A. butzleri* ED-1 and *Arcobacter* L

AMM was capable of supporting electrogenic growth and that the carbon source used had no significant effect on the redox potential generated with cultures using acetate and lactate as their sole carbon source having similar redox potentials over a 120 h time period, with redox potentials declining at a similar rate and both cultures reaching the same end point redox potential of -225 mV after 120 h. However this experiment was only performed once and as such must be repeated in order to confirm whether this result is representative of *A. butzleri* ED-1 growth in a half cell with AMM as the basal medium.

This particular culture of *A. butzleri* ED-1 was shown to be more electrogenic than this partcular culture of *Arcobacter* L, This suggested that *A. butzleri* ED-1 is more electrogenic than *Arcobacter* L, which was to be expected due to its association with the electrode (Fedorovitch *et al*., 2009). It must be noted that the experiment was only performed once and redox potentials of pure *Arcobacter* culture can be highly variable and thus this comparison experiment must be repeated at least in triplicate to show whether *A. butzleri* ED-1 consistently generated a more negative redox potential than *Arcobacter* L. It must be noted that these experiments do not show that *A. butzleri* ED-1 is more electrogenic than *Arcobacter* L on the level of individual cells. One possible reason for the more electrogenic nature of *A. butzleri* ED-1 is discussed in chapter 4.

It was concluded that AMM was capable of supporting electrogenic growth of *A. butzleri* ED-1 and that this particular culture was more electrogenic than this particular culture of *Arcobacter* L. Before any further conclusions regarding cultures of *A. butzleri* ED-1 being more electrogenic than cultures of *Arcobacter* L the experiments must be repeated.

3.3.3 Growth of *A. butzleri* ED-1 on different carbon sources

A. butzleri ED-1 exhibited strong growth on acetate, lactate, fumarate and glutamate and weak growth on malate.

The growth of *A. butzleri* ED-1 on acetate and lactate was predicted by metabolic reconstruction (Ler 2009), which suggested that acetate and lactate can be used by *A. butzleri* ED- as its sole carbon source. The higher final yield achieved by bacteria using lactate was also predicted by the metabolic reconstruction, which shows how lactate generates more energy carrying units then acetate (Ler 2009). Two molecules of acetate generated 1 $FADH_2$, 2 NADH and 1 reduced ferredoxin and 2 molecules of lactate generated 7$FADH_2$, 6NADH and 1 reduced ferredoxin. The increased energy yielded from lactate was the most likely explanation for the increased growth of *A. butzleri* ED-1 consistently observed across quadruplicate cultures when 30 mM lactate was serving as the sole carbon source.

Therefore it was experimentally shown that *A. butzleri* ED-1 could use acetate and lactate as a carbon source as predicted by the metabolic reconstruction (Ler 2009). The model in the metabolic reconstruction suggests that lactate yields more energy than acetate, an assertion which is validated by the higher final A_{600} of cultures grown on lactate (see Figure 3.6).

Growth on fumarate was predicted by the metabolic reconstruction and it was shown experimentally that *A. butzleri* ED-1 was capable of strong growth on fumarate. *A. butzleri* ED-1 encodes a bi-directional fumarate reductase and therefore it is capable of oxidising fumarate to malate, thus using it as a carbon source as well as an electron acceptor during anaerobic respiration (Ler 2009). This suggested that *A.*

butzleri ED- 1 can use fumarate as a carbon source under aerobic and micro-aerobic conditions as under anaerobic conditions it would presumably use fumarate as a terminal electron acceptor instead although this has yet to be validated by experimental data.

A. butzleri ED-1 was found to grow strongly when using glutamate as a carbon source, with final A_{600} of 0.95 being reached. The bacterium was found, in quadruplicate, to exhibit a "two stage" growth curve when grown on glutamate. This "two stage" growth curve was observed in all four replicate glutamate cultures so it is not an isolated occurrence and therefore unlikely to be due to a mutation allowing for more efficient utilisation of glutamate. The purity of the cultures was confirmed by light microscopy and plating a sample of the end culture on selective and non-selective agar and as such the growth pattern was not due to contamination. One possible explanation for the two stage growth of *A. butzleri* ED-1. One possible explanation was that glutamate also enhances the growth of *A. butzleri* ED-1. When grown with just glutamic acid present *A. butzleri* ED-1 reached a higher final A_{600} (see Figure 3.3) which suggests it enhances the growth of the bacterium. Therefore the two stage growth could be due to the glutamate enhancing the growth of *A. butzleri* ED-1 on "carry over" nutrients before the bacteria switches to using glutamic acid as its sole carbon source when these "carry over" nutrients are used up. In order to test this a similar experiment to those shown in Figure 3.2 must be performed with glutamate as should experiment where *A. butzleri* ED-1 is cultured with and without glutamate and without any additional carbon source added.

The growth on malate was weaker than expected as according to the metabolic reconstruction data malate yields a higher amount of energy than acetate i.e. malate yields 4 NADH, 1 $FADH_2$ and 1 reduced ferredoxin (Ler 2009); therefore the bacteria cultivated on malate should achieve comparable final yields to acetate as opposed to a final A_{600} of 0.25. The most likely explanation for the weaker growth on malate was due to inefficient uptake of malate by *A. butzleri* ED-1.

Arcobacter spp. were thought to be capable of growth on propanoate and butanoate were they are thought to traverse the methyl-citrate cycle and enter the TCA cycle as succinate and thus serve as a source of energy (Ler 2009). Experimental data showed that *A. butzleri* ED-1 was consistently unable to grow on butanoate and propanoate and that the compounds have some sort of inhibitory effect as the small amount of growth due to carry over nutrients as observed for cultures without a carbon source was not observed. One probable explanation for the apparent inhibitory effects of propanoate and butanoate was acidification of the basal medium by the stock solution of propanoate or butanoate which would prevent *A. butzleri* ED-1 growing if they reduced the pH of the medium to below 5.5 as *A. butzleri* ED-1 was previously shown not to grow at pH less than 5.5 (data not shown). Therefore before the lack of growth on propanoate/ butanoate can be confirmed the experiment must be repeated with the stock solutions used adjusted to a pH of 6.5-7. As such the ability of *A. butzleri* ED-1 to utilise propanoate/ butanoate as its sole carbon source remains unknown.

A. butzleri was also found to be incapable of growing on ethanol and glycerol which is unsurprising as the bacterium lacks enzymes such as alcohol dehydrogenase which would be required to utilise these carbon sources. The bacterium was unable to use formate as a carbon source, despite being able to use formate as an electron acceptor under anaerobic conditions due to the presence of a formate dehydrogenase (Miller *et al*., 2007 Ler 2009), which suggests that the formate dehydrogenase is not bidirectional.

The bacterium was found consistently to be incapable of growing on citrate which supports genomic data and metabolic reconstruction, which predicted that direct entry of citrate into the TCA cycle does not yield acetyl CoA to the citramalate cycle and therefore citrate cannot be used to generate biosynthetic precursors (Ler 2009). Therefore this experiment showed that the metabolic reconstructions assertions regarding citrate's unsuitability as a carbon source is correct.

A. butzleri was found to be incapable of growing on ethanol and glycerol. The bacterium was unable to use formate as a carbon source, despite being able to use formate as an electron acceptor under anaerobic conditions due to the presence of a formate dehydrogenase (Miller *et al*., 2007, Ler 2009), which suggests that the formate dehydrogenase is not bidirectional, although this was not confirmed experimentally. The inability to use ethanol or glycerol as a carbon source is somewhat surprising as *A. butzleri* ED-1 was found to encode for alcohol

dehydrogenases and therefore should have been able to convert ethanol into acetaldehyde and derive one molecule of NADH and therefore support a low level of growth (Ler 2009). Therefore the lack of growth on ethanol was most likely due to the enzymes being inactive or unexpressed.

A. butzleri ED-1 was predicted to be unable to use glucose as its sole carbon source due to the lack of 6-phosphofructokinase, hexokinase, glucokinase a glucose uptake system (ler 2009). However growth of *A. butzleri* ED-1 was not.

In order to expand on all these experiments they should be repeated on *Arcobacter* L in order to validate the metabolic reconstruction of that bacterium and experimentally compare what carbon sources the two species of *Arcobacter* were capable of growing on.

Therefore it can be concluded that *A. butzleri* ED-1 shows strong growth on acetate, lactate, fumarate and glutamate and weak growth on malate. It was also concluded that the metabolic reconstructions assertions with regards to these carbon source utilisation were correct and it was reasonable to assume that the predicted pathways were functional. The prediction that *A. butzleri* ED-1 could use propanoate as a carbon source was not supported by this set of experimental data, However the inability of *A. butzleri* ED-1 cannot be confirmed without a repeat of this experiment. It was also concluded that *A. butzleri* ED-1 cannot use formate, ethanol or glycerol as its sole carbon source.

3.3.4 The development of an *A. butzleri* ED-1- *E. coli* binary culture for the electrogenic degradation of glucose

It can be seen from Figure 3.7 that *A. butzleri* ED-1 rapidly proliferated within the binary culture and established itself as the dominant species within the culture after 8 h of growth and that the number of *E. coli* did not significantly increase within the 24 h time period. The consumption of glucose within the first 8 h suggests that the *E. coli* is fermenting the glucose, which would correlate with the small rise in CFU/mL of *E. coli* within the first 8 h ($2.7 *10^6$ to $4.0 *10^6$) however after that *A. butzleri* ED-1 feeds on the acetate generated and rapidly out competes the *E. coli*. This was to be expected as the basal AMM is optimised for *A. butzleri* ED-1 growth and *E. coli* is unable to use acetate anaerobically. The numbers of *A. butzleri* ED-1 decline after 8 h dropping from$4.2 * 10^7$ to $2.0 *10^7$ from 8 h to 24 h, which is presumably due to consumption of acetate and the batch nature of the system meant no fresh nutrients were supplied., although this could not be confirmed without measuring the levels of acetate first. The lack of reduction in redox potential was surprising as pure cultures of *A. butzleri* ED-1 reach a much more electronegative redox potential in a similar time frame i.e. typically around -1 mV to -100 mV depending on incubation temperature, prior deoxygenating of the basal medium etc. It must be noted that in pure culture experiments acetate (or another carbon source) was always supplied in excess (typically 30 mM). Therefore the reduced rate of generation of an electronegative redox potential is most likely due to *A. butzleri* ED-1 cell death due

to depletion of acetate. One possible way to investigate this would be to repeat the experiment with batch feeding or to repeat the experiment with a higher amount of glucose.

It was therefore concluded that when grown as a batch culture for 24 h *A. butzleri* ED-1 out competes *E. coli* B, feeding off acetate generated by fermentation of glucose by *E. coli* B within the first 8 h of incubation. It was also concluded that the mixed culture does not generate as an electronegative redox potential as a pure culture of *A. butzleri* ED-1.

The dynamics of the binary culture were further investigated by culturing the bacterium under continuous flow culture conditions.

The redox potential was considerably less than that of pure culture *A. butzleri* ED-1 which typically reaches redox potentials of -200 mV with the lowest redox potential reached within the system being -30 mV. The lack of a highly electronegative redox potential after 300 h suggests that the culture is not capable of degrading glucose in a fashion that is significantly electrogenic. A large spike in number of *E. coli* and *A. butzleri* ED-1 was observed after there were several possible reasons for the poor performance of the system.

The system was not operated under strictly anaerobic conditions and was subject to repeated sample and so could have easily become oxygenated. Oxygenation of the system would lead to a weakly electronegative redox potential as the bacteria in the system would respire using the oxygen in system in the system in preference to the anode. Increased oxygen in the system would also cause an increase in *E. coli* as the bacteria would oxidativley metabolise the glucose which would result in increased growth as opposed to fermentation of the glucose which would occur under anaerobic conditions. However oxidative metabolism of glucose would mean the end products of glucose metabolism i.e. acetate, formate and ethanol would not be produced, which means *A. butzleri* ED-1 would not have a carbon source to use and so could not have experienced an increase in number after 150 h of growth, unless it was utilizing glucose which, was predicted to be impossible due to lacking several key genes. In order to investigate this theory the continuous culture experiment would need to be repeated under more stringent anaerobic conditions.

The large spike in *E. coli* was potentially due to the bacteria adapting better to the medium (which was optimised for *Arcobacter* growth) and to the MFC environment Pervious studies showed that *E. coli* have been found to adapt to MFCs (Zhang *et al*., 2006) although in that study the half-cell generated a much more electronegative redox potential of -200 mV (Zhang *et al*., 2006). It must be noted that this experiment was performed in a single half MFC set up and so the increase in *E. coli* may have been due to experimental error. In order to confirm whether *E. coli* adapted to the MFC environment this experiment would need to be repeated with several half MFC set ups.

Alternatively the lack of *A. butzleri* ED-1 and could be due to the mixed acid fermentation of glucose by *E. coli*. *E. coli* can produce acetate, lactate, succinate, formate and ethanol from the fermentation of glucose (Madigan and Martinko 2006). *A. butzleri* ED-1 has been predicted to use formate as terminal electron acceptor (Ler 2009); therefore the lack of electrogenic glucose degradation could be due to the bacterium using formate as a terminal electron acceptor as opposed to the anode. One possible experiment would be to use a homoglucose fermenter such as *Clostridium thermoacetum*, which converts 1mol glucose to 3 mol. acetate by fermentation (Drake 1982). This would show whether or not the formate produced by *E. coli* glucose fermentation was having a detrimental effect on electrogenesis by *A. butzleri* ED-1. The use of homolactate fermenters may also improve the electrogenicty of the culture as *A. butzleri* ED-1 was shown to exhibit stronger growth on lactate than acetate. These experiments would require further growth experiments to alter the basal AMM to support the growth of a homoglucose or homolactate fermenter as well as *A. butzleri*. If the *A. butzleri* ED-1 was using the formate produced by *E. coli* as a terminal electron acceptor changing the glucose fermenter/ switching to a homolactate fermenter would yield a more negative redox potential. This hypothesis could also be tested by culturing A. *butzleri* ED-1 using formate as a terminal electron acceptor and measuring the growth.

It was concluded that this particular binary culture setup was was not capable of electrogenically degrading glucose, although further work is required to determine

whether or not a binary culture containing *A. butzleri* ED-1 would be viable for electrogenic glucose degradation and thus could be subsequently used for electrogenic cellulose degradation.

3.4 Conclusions

It was concluded that minimal medium can successfully support the growth of *A. butzleri* ED-1 meaning that experiments involving fluorescent imaging can be performed in future as can a wide variety of growth experiments on different substrates. The traditional approach of omission experiments used here was the first time that this, or indeed any approach, has been used for the creation of a minimal medium for *Arcobacter spp*. growth.

The metabolic reconstruction data has been validated with experimental evidence that *A. butzleri* ED-1 can grow using a variety of organic acids as its sole carbon source and does not require additional amino acids for growth.

The ability of *A. butzleri* ED-1 to use Fe^{3+} and Mn^{4+} as insoluble terminal electron acceptors remains unknown and further investigation in this area is required.

A binary culture of *A. butzleri* ED-1 and *E. coli* is incapable of degrading glucose in an electrogenic fashion either due to it being outcompeted *by E. coli*, using formate produced by *E. coli* as a terminal electron acceptor as opposed to the anode or due to oxygenation. Further experiments need to be performed to determine whether an *A. butzleri* based binary culture can be used to electrogenically degrade glucose.

With these conclusions in mind it was decided to use the AMM created to culture *A. butzleri* ED-1 and *Arcobacter* L on a half MFC anode and glass coverslips in order to investigate biofilm formation, discussed in chapter 4.

Chapter 4

Fluorescent imaging of *Arcobacter butzleri* ED-1 and *Arcobacter* L on the anode of a microbial fuel cell

4.1 Introduction

As discussed in chapter 1 the formation of an anodic biofilm has been found to be an essential part of electrogenesis by *Geobacter sulfurreducens* (Regurea *et al*., 2005, Franks *et al*., 2008, Nevin *et al*., 2009) and *Shewanella oneidensis* MR-1 (Gorby *et al*., 2006). This is discussed in detail in chapter 1; however in brief; in an anodic biofilm the bacteria produce an electrically conductive extracellular matrix consisting of c type cytochromes and electrically conductive pili known as nanowires, which facilitate the transfer of electrons from bacteria to the anode, allowing thick biofilms to form on the anode and a strong current to be generated.

The anodic biofilm is just one example of biofilm formation as such biofilms and biofilm formation are discussed in detail in section 4.1.1-4.1.2.

4.1.1 What is a biofilm?

A biofilm is an aggregation of cells with an extracellular matrix of highly hydrated extracellular polymeric material (EPS) which consists of carbohydrates, proteins, lipids and DNA (Flemming and Wingender 2010). Biofilms can consist of a homogenous or heterogeneous mix of bacteria and examples of biofilm formation have been found throughout the archeal and bacterial kingdoms (Stoodley *et al*., 2004).

Biofilms are structurally heterogeneous, highly dynamic and competitive environments with intense cell-cell communication, horizontal gene transfer and physioChemical gradients occurring within the biofilm (Flemming and Wingender 2010).

The formation of biofilms allows for bacteria to adapt and thrive in any number of diverse environments and dispersal of a biofilm provides a source of bacteria to colonise other niches within an environment (Stoodley *et al*., 2004). Biofilms are particularly effective at providing resistance to a number of stresses that would be deleterious to microbial growth (Stoodley *et al*., 2004. The formation of a biofilm has been found to protect against a number of adverse conditions such as heavy metal toxicity; it was found that biofilm associated cells were between 2- 600 times more resistant to heavy metal toxicity then planktonic cells (Teitzel and Parsek 2003). Biofilms have also been found to convey resistance to acidic conditions; in one particular study, 43.3% of biofilm associated bacteria survived prolonged exposure to killing pH i.e. pH 3.5 (McNeill and Hamilton 2004). Biofilms also provide a capacity to survive dehydration (Le Magrex-Debar *et al*., 2000), UV radiation (Espeland *et al*., 2001) and phagocytosis (Leid *et al*., 2002).

Biofilms have been found to convey a resistance to antibiotics, which is of particular importance from a medical standpoint as they are of major importance to chronic infections such as those associated with implanted medical devices (Stewart and Consterton 2001). The EPS of the biofilm prevents antibiotic penetration, which lessens the effectiveness of antimicrobial agents (Stewart and Consterton 2001) and

several other factors of the biofilm also lead to an ineffectively of antimicrobial agents. These factors include a high rate of cell-cell communication and horizontal gene transfer which allows bacteria in a biofilm to be become better adapted to antibiotics and the formation of persister cells; dormant varieties of regular cells that are highly resistant to antibiotics and so can re-colonize following treatment of a biofilm by antimicrobial agents (Lewis 2010). The antimicrobial nature of biofilms has received considerable study but is far beyond the scope of this project.

Therefore biofilm formation is a response by bacteria to a range of environmental stresses and changes allowing them to survive, thrive and colonize a wide range of environments.

The currently accepted model of biofilm formation has been developed by the use of fluorescent microscopy techniques and the use of model organisms such as *Psuedomonas aeruginosa and Bacillus subtilis* to better understand the cellular and molecular processes involved. These techniques are discussed in section 4.1.2.

4.1.2 The formation of biofilms

There have been two main approaches to studying the formation of biofilms and developing the currently understood model of biofilm formation; the use of fluorescent microscopy and a study of various cellular and molecular processes involved.

4.1.2.1 Fluorescent microscopy

Fluorescent microscopy has proved an invaluable tool in the study of biofilms (Costerton *et al*. 1994) as the technique allows for non-destructive imaging of live, hydrated and intact biofilms thereby giving a more accurate picture of the spatial arrangement of cells within a biofilm (Lawrence and Neu *et al*. 1999). This has the distinct advantage over electron microscopy based techniques as it allows the biofilm to be imaged while 'intact' although fluorescent microscopy techniques lack the magnification that EM work provides (Surman *et al*., 1996).

While providing understanding of the morphological and structural changes that occur during biofilm formation and growth, fluorescent microscopy does not provide understanding of the cellular and molecular processes involved in biofilm formation.

To better understand these processes organisms, such as *Pseudomonas spp.*, and *B. subtilis* have been extensively studied.

4.1.2.2 Cellular and molecular processes of biofilm formation

Work on a variety of different species has allowed for a generalized model for the cellular processes involved in bacterial biofilm formation to be developed (Lemon *et al*., 2008). In essence biofilm formation occurs when bacteria undergo a regulated physiological switch from a motile unicellular state to a sedentary multi-cellular state

(Lemon *et al*., 2008). Biofilm formation first occurs when cells weakly attach to a surface by non-specific attractions such as Van Der Walls forces. Bacteria then begin producing adhesins that more firmly anchor cells to the surface (Gotz 2002). In motile species of bacteria flagella are critical for localizing the bacteria to the surface, although once localized the bacteria will rapidly lose motility The importance of flagella to initial localization and attachment is shown by a large number of studies where flagella deficient mutants show an inhibited capacity for biofilm formation (Pratt and Kolter 1998, Watnick and Kolter 1999, Lemon *et al*. 2008, Joshua *et al*., 2006). The role of flagella in biofilm formation is discussed in Chapter 5. The adhesins produced also enable the cells to attach to other cells, with many adhesins, such as the Bap in *Streptococcus spp or* EspA *in E. coli*, attracting and actively promoting cell-cell attachment, resulting in the formation of microcolonies (Moreia *et al*., 2006, Lembke *et al*., 2006).

Following the aggregation of cells into micro-colonies the cells begin to produce EPS. EPS production involves a complex series of genetic switches with certain genes being up-regulated and others being down-regulated (Lemon *et al*., 2008). For example in *B. subtilis* biofilm formation the SinR protein, which represses the genes responsible for EPS production in the bacteria is antagonized, which leads to the expression of Eps, TasA and YqxM which then leads to the production of EPS (Kearns *et al*., 2005, Branda *et al*., 2006). Other bacteria have their own particular genetic switches and methods to produce EPS. EPS allows the bacteria to aggregate into large macro-colonies, which as previously described are large, heterogeneous

structures with considerable physiological and genetic diversity in the population observed.

The final stage of biofilm life cycle is dispersion where cells switch from biofilm to a planktonic state of being. Dispersion is an important part of the biofilm life cycle as it allows the cells to colonize new environments. It must be noted that cells are continually attaching to and dispersing from a biofilm (Lemon *et al*., 2008). Complete dispersal of a biofilm can be due to a number of different factors such as starvation, acid stress and decreased oxygen tension; these are discussed more fully in section 4.4

Using the information gained from imaging studies and studies of the cellular and molecular processes of biofilm formation, the following model of biofilm formation has been developed; this is illustrated in Figure 4.1.

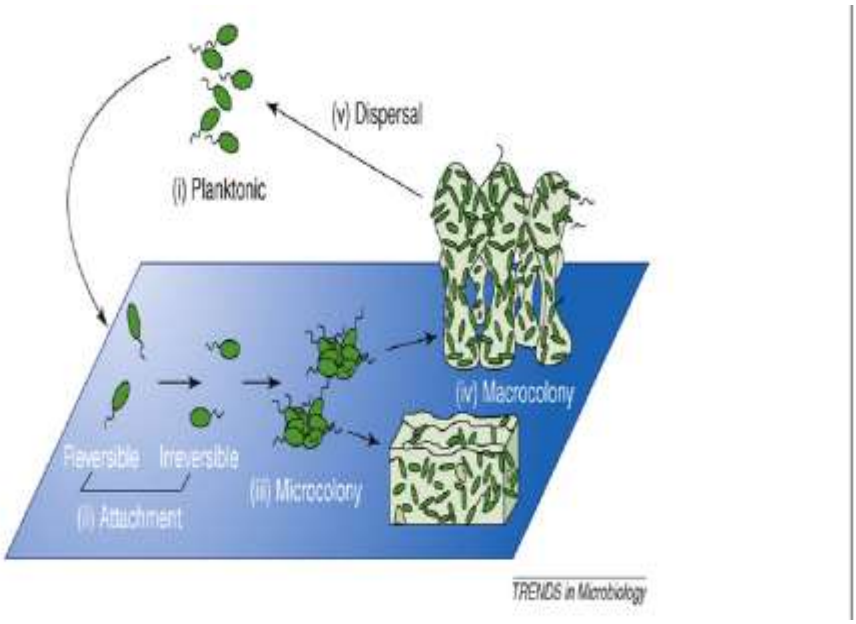


Figure 4. 1 taken from Monds and O'Toole 2009 it shows the accepted model of biofilm formation developed by the use of fluorescent microscopy and the study of a the cellular and molecular processes of a variety of biofilm forming bacteria. Cells initially attach to a surface by weak molecular interactions such as Van Der Walls forces and hydrogen bonds. Then the cells produce adhesins and/or localise together by use of flagella (if motile) which more firmly attaches the cells to the surface and promotes cell-cell adhesion resulting in the formation of a micro-colony. The bacteria in the micro-colony then undergo a series of genetic switches that results in the production of EPS which aggregates the cells together, forming macro-colonies, large collections of highly differentiated, heterogeneous cells. The final stage is dispersion where cells revert to a planktonic state, which occurs naturally with cells dispersing and joining the biofilm continually and can also be caused by environmental stresses.

4.1.2 The Importance of anodic biofilms in MFC

As discussed in chapter 1 (section 1.6.2) the formation of an anodic biofilm is an important part of electrogenesis by a wide variety of electrogenic bacteria. Bacteria that form an anodic biofilm tend to be distinct from those that use mediators to perform electrogenesis. Examples of bacteria that form an anodic biofilm are *Desulfovibrio desulfuricans, Ochrobactrum anthropi* YZ-1 and *Geobacter metallireducens* which were all isolated from anodic biofilms (Logan 2009, Zuo *et al*., 2008 and Bond *et al*., 2002). By contrast examples of bacteria that perform electrogenesis by mediator include *Psuedomonas aeruginosa* and *Geothrix fermentans* (Rabaey *et al*., 2002, Logan 2009). *Shewanella oneidensis* is something of an exception as it is thought to use both mediator compounds and an anodic biofilm to perform electrogenesis (Lanthier *et al*., 2008, Marsili *et al*., 2008) where flavins play a role in the transfer of electrons through the anodic biofilm and in transferring electrons to the anode from bacteria growing in the planktonic phase of the MFC (Lanither *et al*., 2008, Marsili *et al*., 2008). This is discussed in more detail in chapter 1.

The anodic biofilm of *Geobacter sulfurreducens* is the most well characterised anodic biofilm with the most important study in the context of this project is the work by Franks *et al*., 2008. This particular study used a specifically constructed MFC to image fluorescently tagged *Geobacter sulfurreducens* in real time as they colonised the anode and formed a thick biofilm with an electrically conductive extracellular matrix. The study showed that *Geobacter sulfurreducens* cells form a

50 μm thick biofilm that produces a current of 0.21 mA and that an accumulation of protons (i.e. a decrease in pH) due to oxidation of organic matter can limit current production due to inhibiting cell growth (Franks *et al,*. 2008) . A mature *Geobacter sulfurreducens* biofilm based on the results of this study and subsequent studies is shown in Figure 4.2.

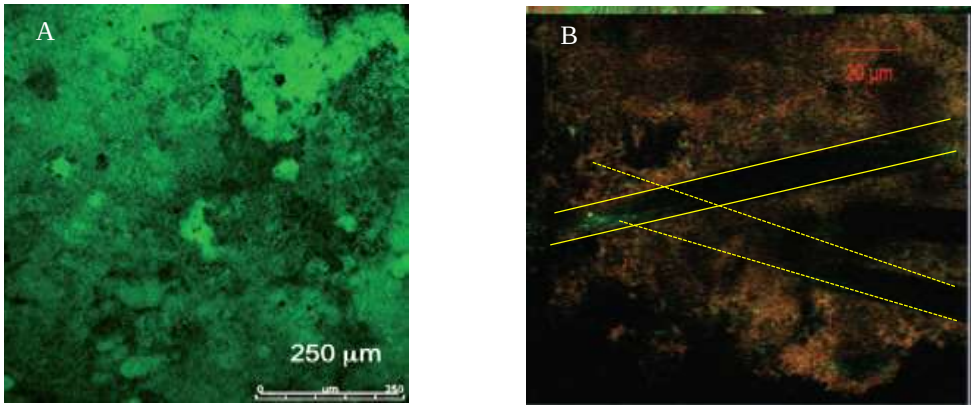


Figure 4.2 adapted from Franks *et al*., 2008 (A) and Nevin *et al*., 2008 (B). Both images were taken from a mature *Geobacter sulfurreducens* biofilm grown in a mini MFC specifically designed for imaging. Images were taken by confocal laser scanning microscopy. Image A) was taken after 400 h of growth when the current was measured at 0.21 mA in the X-Y focal plane (Franks *et al*., 2008). Image B was taken in Z- X axis and shows a mature biofilm of *Geobacter sulfurreducens* extending from an anode fibre (outlined in yellow). It shows the biofilm extending 30- 50 μm from the anode, illustrating the thickness of a mature *Geobacter sulfurreducens* biofilm. The biofilm is supported due to a conductive extracellular matrix consisting of c type cytochromes (OmcZ) and nanowires which facilitates the transfer of electrons over a large distance, thus allowing all the bacteria of the biofilm to use the anode as an electron acceptor.

This and other studies show the potential importance of anodic biofilm formation in electrogenesis and therefore it was decided to study anodic biofilm formation by *A. butzleri* ED-1 and *Arcobacter* L.

4.1.3 Aims and Objectives.

Biofilms of *A. butzleri* ED-1 and *Arcobacter* L were cultured on glass coverslips and the anodes of half MFC over a period of 120 h i.e. the time it takes for a stable redox potential to develop in an *A. butzleri* ED-1 half MFC. This was done to investigate the following areas

- To compare biofilm formation by a pure culture of *A. butzleri* ED-1 and *Arcobacter* L both in a half MFC environment and on a glass coverslip under microaerobic conditions. This was done to show if there were any differences in biofilm formation between the two species and if said differences corresponded to the differences in electrogenic ability previously observed by the two cultures i.e. did *Arcobacter* L form a less populous biofilm than *A. butzleri* ED-1 with it being the less electrogenic of the two species.
- To compare *Arcobacter spp*. anodic biofilm formation to anodic biofilm formation by other electrogenic bacteria. This would show whether *Arcobacter spp*. anode interaction was significantly different from more well characterised electrogenic bacteria such as *Geobacter sulfurreducens* and *Shewanella oneidensis* and therefore perhaps suggests a new model for electrogenesis if *Arcobacter spp*. anodic biofilm formation was found to be significantly different.

- A mixed culture of *A. butzleri* ED-1 and *Arcobacter* L was grown on glass coverslips to see whether co-culture affects biofilm formation by the bacteria.

4.2 Results

4.2.1 The construction of pJK1 and pMK1

In order to perform fluorescent imaging of *A. butzleri* ED-1 and *Arcobacter* L, a vector that constitutively expressed fluorescent protein was required. The GFP plasmid suitable for expression in *Arcobacter spp*. was constructed by amplifying a 5kb region of pWM1007, a GFP expressing *Campylobacter spp*. vector (Miller *et al*. 2000) and fusing it to a 2 kb fragment of an *Arcobacter* cryptic plasmid, which contained the *repB* gene for *Arcobacter* replication isolated from *Arcobacter spp.* found on broiler carcasses (Harrass *et al*., 1998).The CFP vector was created by excisng the CFP cassette from pWM1009 (Miller *et al*. 2000) by digestion with *Nco*I and *Bst*BI and ligating into pJK1 i.e. the GFP expressing plasmid (Kolmar 2009). These vectors were chosen as expression of the fluorescent proteins was constitutive and under control of the putative promoter sequence which was found to be functional in both *E. coli* and *C. jejuni* (Woston *et al*., 1998). Due to the close relationship between *Arcobacter spp*. and *Campylobacter spp*. it was assumed the promoter would be able to control expression in *Arcobacter spp*. A schematic diagram of the plasmid is shown in Figure 4.3.

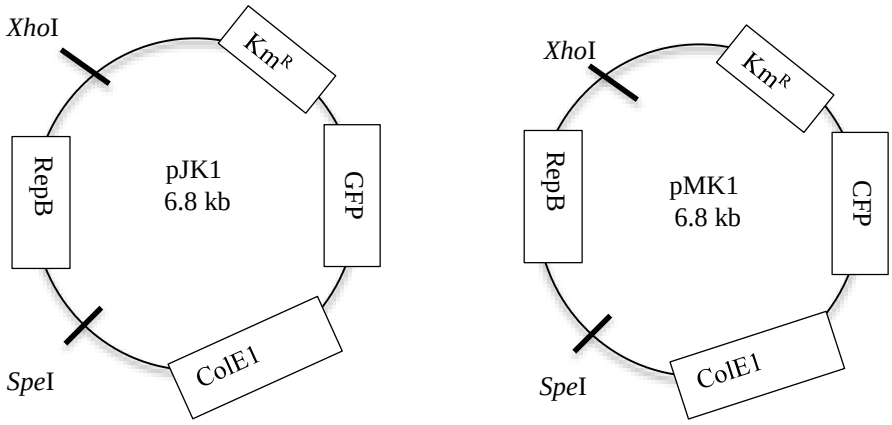


Figure 4. 3 shows a schematic diagram of pJK1 (Kolmar 2009) and pMK1. Both plasmids are 6.8 kb and contain origins of replications for *E. coli* (ColEI) *Arcobacter* (RepB), a kanamycin resistance cassette and a fluorescent protein cassette either GFP or CFP. The fluorescent protein cassette is under the control of a *Campylobacter* promoter that constitutively expresses in both *Arcobacter* and *E. coli* (Wosten *et al*., 2000). The plasmid possess and RK2 *ori*T which allows it to be mobilised from *E. coli* to *A. butzleri* ED-1 and *Arcobacter* L by triparental mating using the helper plasmid pRK600.. The *Rep*B gene for replication in *Arcobacter spp*. comes from an *Arcobacter* cryptic plasmid isolated from *Arcobacter spp*. on broiler carcasses (Harrass *et al*., 1998).

Sanger sequencing was used to verify each construct. The vectors were also confirmed by restriction digest with *EcoRI* or *NcoI* to give a single linear fragment as shown in Figure 4.4.

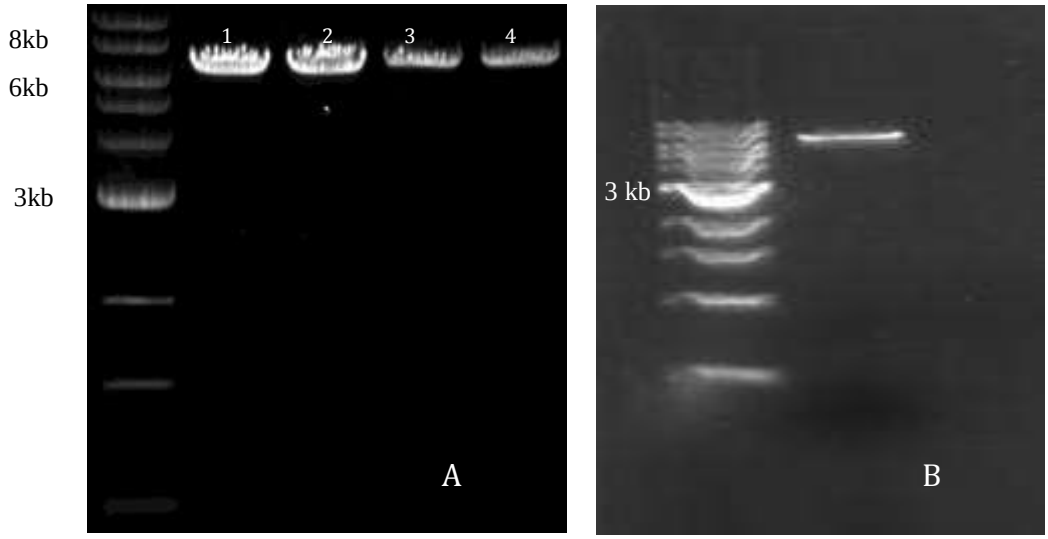


Figure 4.4 shows a restriction digest of pJK1 (image A) with *Eco*RI (Kolmar 2009) and pMK1 (image B) with *Nco*I. Gels were 1% (w/v) agarose ran with 1* TBE buffer for 1 h at 120 V. The protocol for restriction digest is given in chapter 2. Lanes 1-4 in image A) show a single 7 kb band of pJK1 linearized by restriction digest with *EcoR*I. Image B lane 1 shows a 7 kb band of pMK1 linearised by digestion with *Nco*I. The difference in restriction enzymes used was due to availability of enzymes at time of cloning. The sizes were measured using a 1 kb ladder from NEB. Sanger sequencing was then used to confirm whether or not the constructs were correct.

Sequencing and restriction digests both confirmed that the pJK1 and pMK1 samples obtained were in fact the correct constructs. These constructs were successfully introduced into *A. butzleri* ED-1 and *Arcobacter* L by triparental mating and shown to express GFP or CFP at sufficient quantities to be visualised by fluorescent microscopy. The introduction of DNA into *Arcobacter* required several different attempts and approaches due to a lack of information regarding the introduction of exogenous DNA into *Arcobacter spp*. The development of a strategy to introduce pJK1 and pMK1 to *Arcobacter spp*. is discussed in section 4.2.2

4.2.2 The introduction of pJK1 and pMK1 into *Arcobacter spp.*

There has been relatively little genetic work performed on *Arcobacter spp*. with the best example being the work by Ho *et al*., who constructed Fla A and FlaB deletion mutants in *A. butzleri* LMG (Ho *et al*., 2008). It was decided to subject the bacteria to a number of different methods for introducing exogenous DNA in order to see which was the most effective. The methods used were

- Electroporation. Electroporation had previously been used b Ho *et al*. to introduce a suicide vector into *A. butzleri* LMG in order to create flagella deletion mutants. The method here used an ice-cold solution of 20 % (v/v) glycerol and 7 % (w/v) sucrose to make *A. butzleri* cells electro competent and then used a voltage of 2.5 kV to electroporate with exogenous DNA (Ho *et al*., 2008).
- Triparental mating. A triparental mating set up using the helper plasmid pRK600 was used to mobilise pJK1 and pMK1 from *E. coli* DH5α into *A. butzleri* ED-1 and *Arcobacter* L. The plasmids were mobilised by the RK2 *ori*T they possessed. Triparental mating with the bacteria in exponential phase and stationary phase was attempted. The protocol for mating and recovery of recombinant *Arcobacter* is given in chapter 2.
- Heat shock. The viability of a heat shock based method using chemically competent *Arcobacter spp*. was investigated. *Arcobacter spp*. were made chemically competent by using ice-cold 0.1 M $CaCl_2$ and transformed by heat shock at 37 ◦C (as opposed to 42 ◦C for *E. coli)*, although otherwise the same

protocol was employed, with a longer growth phase to accommodate the slower growth rate of *Arcobacter spp*. The protocol for creating competent *E. coli* is detailed in chapter 2.

- One step transformation. One step transformation is another method of making chemically competent cells by the use of a transformation and storage solution (TSS solution) developed by Chung *et al*. (Chung *et al*., 1989). TSS solution consisted of LB broth containing 10 % (w/v) polyethylene glycol, 5 % (v/v) dimethyl sulphoxide and 50 mM Mg^{2+} (Chung *et al*., 1989). The TSS solution makes chemically competent cells that can be transformed without a heat shock step, making it useful for the transformation of heat sensitive strains (Chung *et al*., 1989) although the use of a heat shock stage did not decrease the efficiency of transformation (Chung *et al*., 1989). For use with *Arcobacter* Vandamme medium as opposed to LB broth was used. *Arcobacter* were made competent as per the protocol given in the paper, although growth times were adjusted to accommodate the slower growth rate of *Arcobacter.* Chemically competent *Arcobacter* prepared by this method were then transformed with pJK1 or pMK1 with and without heat shock.

These different methods were used on *A. butzleri* ED-1, *A. butzleri* RM4018, *A. butzleri* LMG and *Arcobacter* L. The effectiveness of each method for each strain of *Arcobacter* is shown in Table 4.1.

Method of introducing DNA	*A. butzleri* ED-1	*Arcobacter* L	*A. butzleri* RM4018	*A. butzleri* LMG
Electroporation (Ho *et al*., 2008)	-	-	+	-
One step transformation (Chung *et al*. 1989)	-	-	-	-
One step transformation with heat shock	-	-	-	-
Heat shock/chemically competent cells	-	-	-	-
Triparental mating in stationary phase	-	-	-	-
Triparental mating in exponential phase	+	+	+	+

Table 4.1 shows the success of different methods of introducing exogenous DNA into *A. butzleri* ED-1, *A. butzleri* RM4018, *A. butzleri* LMG and *Arcobacter* L. Electroporation, heat shock, one step transformation and triparental mating in the stationary and exponential phase of growth were attempted. Triparental mating in the exponential phase of growth was the only method that met with success in all four strains although further purification only led to the recovery of *A. butzleri* ED-1 and *Arcobacter* L containing pJK1 or pMK1. Electroporation succeeded in introducing pJK1 *into A. butzleri* RM4018 yielding a single recombinant colony.

Table 4.1 shows that only triparental mating with bacteria in the exponential phase of growth yielded positive results and only *A. butzleri* ED-1 and *Arcobacter* L yielded recombinant cells when subject to further purification. Electroportation of *A. butzleri*

RM4018 yielded successful transformants whereas electroporation of other strains did not. The presence of a type I restriction system in *A. butzleri* ED-1 accounts for the difference between RM4018 and ED-1 but the lack of success in *Arcobacter* L and LMG cannot be explained by restriction (the bacterium does not possess a functioning restriction system). The lack of success in LMG was unexpected as this was the strain used in the Ho *et al*. paper that was successfully electroporated to create flagella deletion mutants.

Following introduction of the plasmid into bacteria presence of the plasmid was shown by gel electrophoresis of cellular lysate (Green *et al*., 2012). This method was used as opposed to pure plasmid obtained by miniprep kit as *A. butzleri* ED-1 and *Arcobacter* L proved resistant to both Qiagen and Promega miniprep kits and their treatment did not yield plasmid. Expression of GFP and CFP at a detectable level was confirmed by imaging the bacteria. The bacteria were imaged from a pure culture on glass slides (see materials and methods for details on sample preparation). As a negative control wild type bacteria were also cultured prepared and imaged. The images are shown in Figure 4.5.

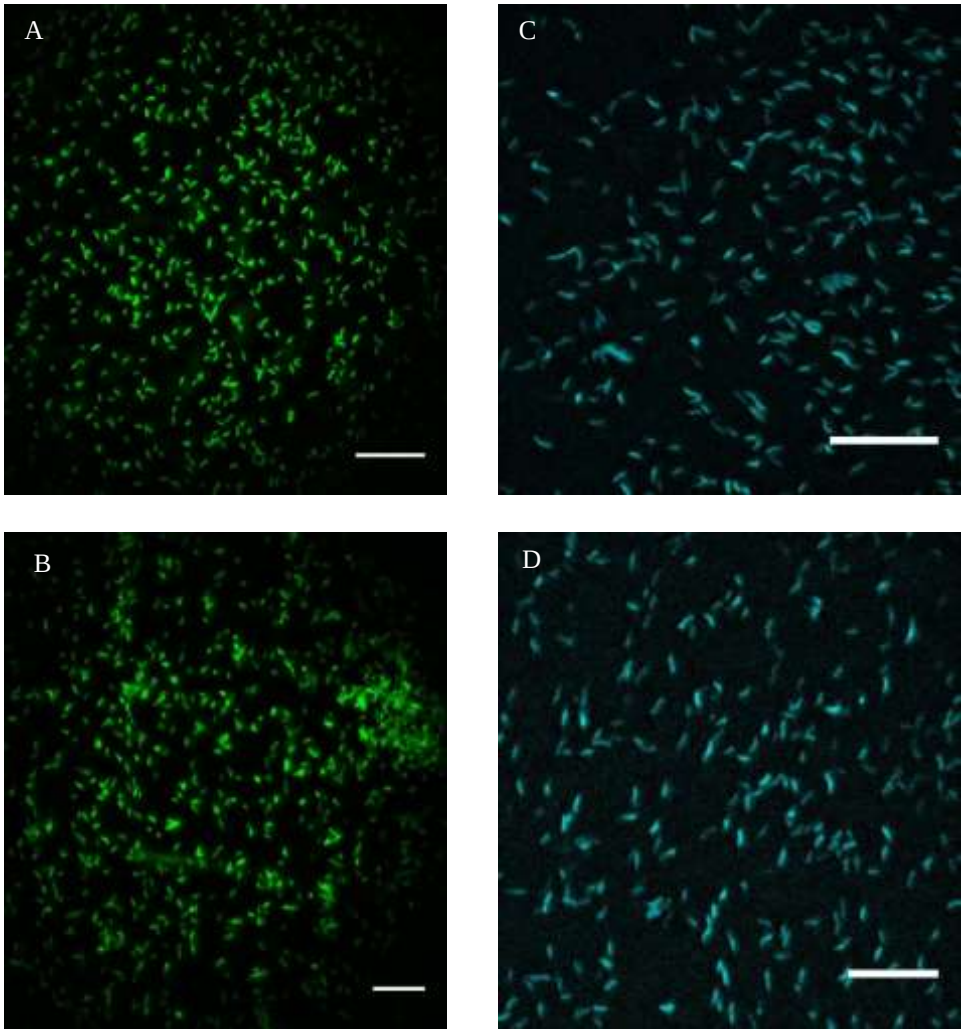


Figure 4.5 shows *A. butzleri* ED-1 (A) and *Arcobacter* L (B) containing pJK1 expressing GFP and *A. butzleri* ED-1 (C) and *Arcobacter* L (D) containing pMK1 and expressing CFP. Scale bar is 10 μm. Imaging was performed using a DeltaVision RT microscope (Applied Precision) with appropriate filters (395 nm-405 nm for GFP and 425 nM-475 nm for CFP), see chapter 2 for full details. Images of the cells were captured using a Cascade2_1K camera (applied precision). Exposure time was 0.8 sec. for GFP expressing cells and 2. 0 sec. for CFP expressing cells, which resulted in the "fuzzier" images observed for CFP expressing cells. These images show that *Arcobacter spp.* express GFP and CFP at a detectable level. Cells averaged 1.96 ± 0.22 μm in length and 0.31 ± 0.06 μm in width.

The images show that both *Arcobacter spp*. express GFP and CFP to detectable levels. Images of CFP expressing bacteria are 'fuzzier' then those of GFP expressing bacteria as CFP has a narrower excitation-emission spectrum then GFP and are less intense and therefore required imaging with a much longer exposure time of 2 sec. as opposed to the 0.8 sec. required for GFP. Wild type bacteria did not fluoresce when imaged (data not shown), showing that the fluorescence observed was not due to auto fluorescence. Therefore the recombinant *Arcobacter spp*. were shown to express GFP/ CFP at detectable levels, which showed that pJK1 and pMK1 were suitable vectors for use in the fluorescent imaging of *Arcobacter spp*.

4.3. 3The formation of *A. butzleri* ED-1 and *Arcobacter* L biofilms on glass coverslips under microaerobic conditions

To understand the interactions of *Arcobacter* and the anode it was imperative to understand general biofilm formation by *A. butzleri* ED-1 and *Arcobacter* L. *A. butzleri* ED-1 and *Arcobacter* L tagged with GFP were cultured microaerobically in AMM with 30 mM acetate serving as the sole carbon source. Sterilised glass coverslips were submerged in the culture in order to promote biofilm formation. To ensure biofilm formation and any differences observed were due to intrinsic qualities of the bacteria rather than external factors such as nutrient deprivation the medium was changed every day. The cultures were grown statically at 30 °C.

A competition experiment (i.e. *A. butzleri* ED-1 and *Arcobacter* L grown in co-culture subject to same feeding conditions as the pure cultures) was also performed. For the competition experiments *A. butzleri* ED-1 was tagged with GFP and *Arcobacter* L was tagged with CFP.

Every 24 h a coverslip was removed using sterile tweezes and imaged using a DeltaVision RT microscope (Applied Precision) with appropriate filters (395 nm-405 nm for GFP and 425 nm-475 nm for CFP). Images of the cells were captured using a Cascade2_1K camera (applied precision). Each image is of a different 100 μm by 100 μm section of a biofilm formed on a 25 mm by 25 mm glass coverslip. Images were taken in in the X-Y focal planes at a 1000 * magnification. A selection of images taken from this experiment are shown in Figure 4.6.

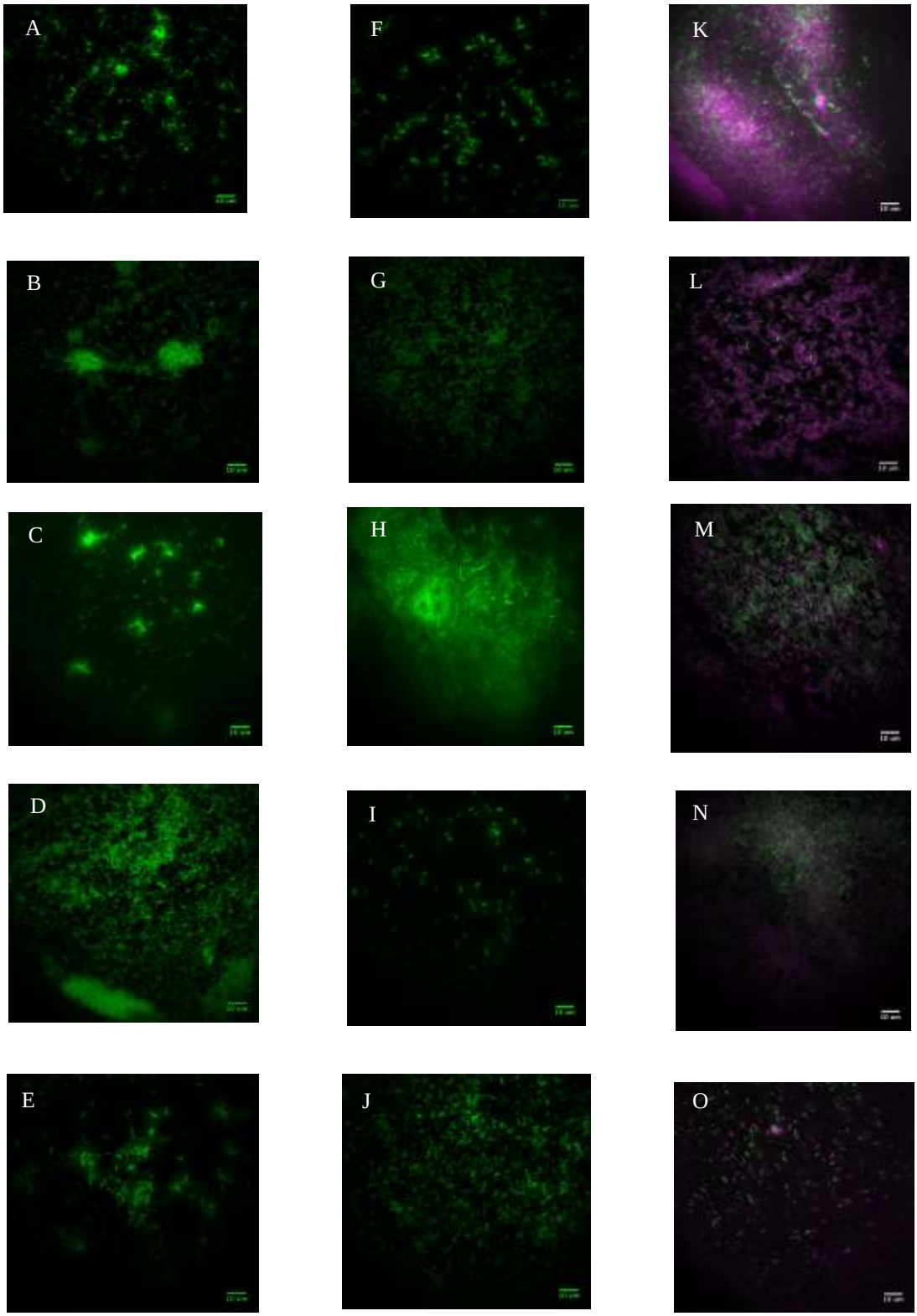


Figure 4.6 shows the formation of an *A. butzleri* ED-1 (A-E), *Arcobacter* L (F-J) and mixed culture (K-O) biofilm on a glass coverslip over a period of 120 h. In mixed culture *A. butzleri* ED-1 tagged with GFP (green) and *Arcobacter* L tagged with CFP (purple). Scale bar represents 10 μm.

The cells were quantified using the cell counter function of the imageJ software. The results are given in table 4.2.

Time (h)	Total cells	% *A. butzleri*	No. microcolonies	No. of cells per microcolony
24	220 ±133/114 ± 40/348 ± 194	25 ± 0.05	4 ± 2/6 ± 0.6	20 ± 13/8 ± 3
48	524 ± 100/850 ± 100/ 566 ± 143	8.58 ± 8.12	5 ± 1/0	29 ± 20/0
72	235 ± 29/491 ± 191/654 ± 129	38 ± 13.2	6 ± 0.6/0	15 ± 9
96	895 ± 249/92 ± 40/1284 ± 200	35 ± 14	0/2 ± 3	0/13 ± 4
120	217 ± 13/454 ± 46/194 ± 45	56 ± 2	4 ± 1/0	18 ± 6

Table 4.2 shows the mean total number of cells in the *A. butzleri* ED-1/*Arcobacter* L/ mixed culture and the % of *A. butzleri* ED-1 in the mixed culture of a 120 h time period. Table 4.2 lists the number of microclonies and cells per micorcolony in the *A. butzleri* ED-1/*Arcobacter* L cultures over a 120 h time period. Numbers were determined from 10 images 100 μm by 100 μm sections of a single biofilm for each culture condition at each taken. Cells counted by use of the imageJ cell counter function

After 24 h *A. butzleri* ED-1 forms a more populous biofilm (220 ± 113) then *Arcobacter* L (114 ± 40) and both biofilms show distinct differences in morphology. *A. butzleri* ED-1 forms a small number of large microcolonies surrounded by individual cells whereas *Arcobacter* L forms a biofilm consisting of smaller more

even distributed micro-colonies and less individual cells. When grown as a mixed population the biofilm is more populace then a pure culture of either species (348 ± 194) with *Arcobacter* L comprising the majority of the population with *A. butzleri* ED-1 forming only 25% of the population.

A biofilm at 48 h exhibited considerable differences in morphology. The *A. butzleri* ED-1 biofilm consists of several large microcolonies (5 ± 1 consisting of 29 ± 20 cells per colony) with smaller aggregates/ individual cells between each colony. The *Arcobacter* L biofilm consists of a single homogenous mass of cells with no microcolonies. The *Arcobacter* L biofilm also has a higher cell population at this stage (850 ± 100 cells as opposed to 524 ± 100 cells for *A. butzleri* ED-1). The mixed species biofilm shows a similar morphology to that of a pure *Arcobacter* L biofilm although it has less cells (566 ± 143) and *A. butzleri* ED-1 comprises only 8.58% of the population.

After 72 h the *Arcobacter* L biofilm has formed into a single large macro colony like structure although it seems to contain less cells then the 48 h old biofilm (491 ± 191 cells). There were also a small percentage of cells (8.3%) with a shorter more rounded appearance displaying greater fluorescence. The cells with an altered morphology were straighter than other *Arcobacter* L. The numbers in the *A. butzleri* ED-1 biofilm had declined from the 48-h time point and a series of small micro colonies had formed (6 microcolonies with 15 ± 9 cells per colonies) with individual cells distributed between them. The morphology of the mixed biofilm remains the

same although the numbers of cells have increased by approximately a 100 and *A. butzleri* ED-1 now comprises 38% of the population.

After 96 h the population of *A. butzleri* ED-1 had dramatically increased (895 ± 249) and the biofilm developed into a homogenous mass of cells indicative of a mature biofilm. By contrast the *Arcobacter* L biofilm had dramatically decreased in numbers (92 ± 40) with most of the cells detaching from the coverslip to leave only a few individual cells and small microcolonies. The mixed species biofilm had once again increased in number (1284 ± 200) with *A. butzleri* ED-1 now comprising 35% of the population. The morphology of the mixed species biofilm was a homogenous mass of cells even distributed across the glass surface.

A number of cells detached from the *A. butzleri* ED-1 biofilm after 120 h leaving a few individual cells and microcolonies (4 ± 1 colonies with 18 ± 6 cells per colony) with the total population of cells decreasing to 217 ± 13 cells. The numbers of the *Arcobacter* L had increased (454 ± 46) from what was observed at 96 h and the micro-colonies had congregated into a thin but homogenous distribution of cells. The mixed species biofilm exhibited a decrease in cell numbers with cells forming a thin, evenly distributed layer across the glass surface. *A. butzleri* ED-1 comprised 56% of the total cell population; meaning that it was now the dominant species by a narrow margin.

4.2.5 The colonisation of a half MFC anode by *Arcobacter spp*. over 120 h

While imaging *Arcobacter spp.* biofilms on glass coverslips provided invaluable information about general *Arcobacter* biofilm formation it did not provide any insight into how *A. butzleri* ED-1 or *Arcobacter* L would interact with the anode over a given length of time. To that end GFP tagged *A. butzleri* ED-1 and *Arcobacter* L were cultivated in a half MFC over 120 h; a 120 h being the time it takes for a stable redox potential to be established. The cells were grown in AMM with 30 mM acetate serving as the sole carbon source as per the set up descried in chapter 2.

Cells were imaged using DeltaVision RT microscope (Applied Precision) with appropriate filters (395 nm-405 nm for GFP and 425 nm-475 nm for CFP). Images of the cells were captured using a Cascade2_1K camera (applied precision). Each image is of a different 100 μm by 100 μm section of a biofilm formed on a section of anodic clothe approximately 10 mm by 10 mm. Images were taken in in the X-Y focal planes at a 1000 * magnification.

The images obtained from this set of experiments are depicted in Figure 4. 7.

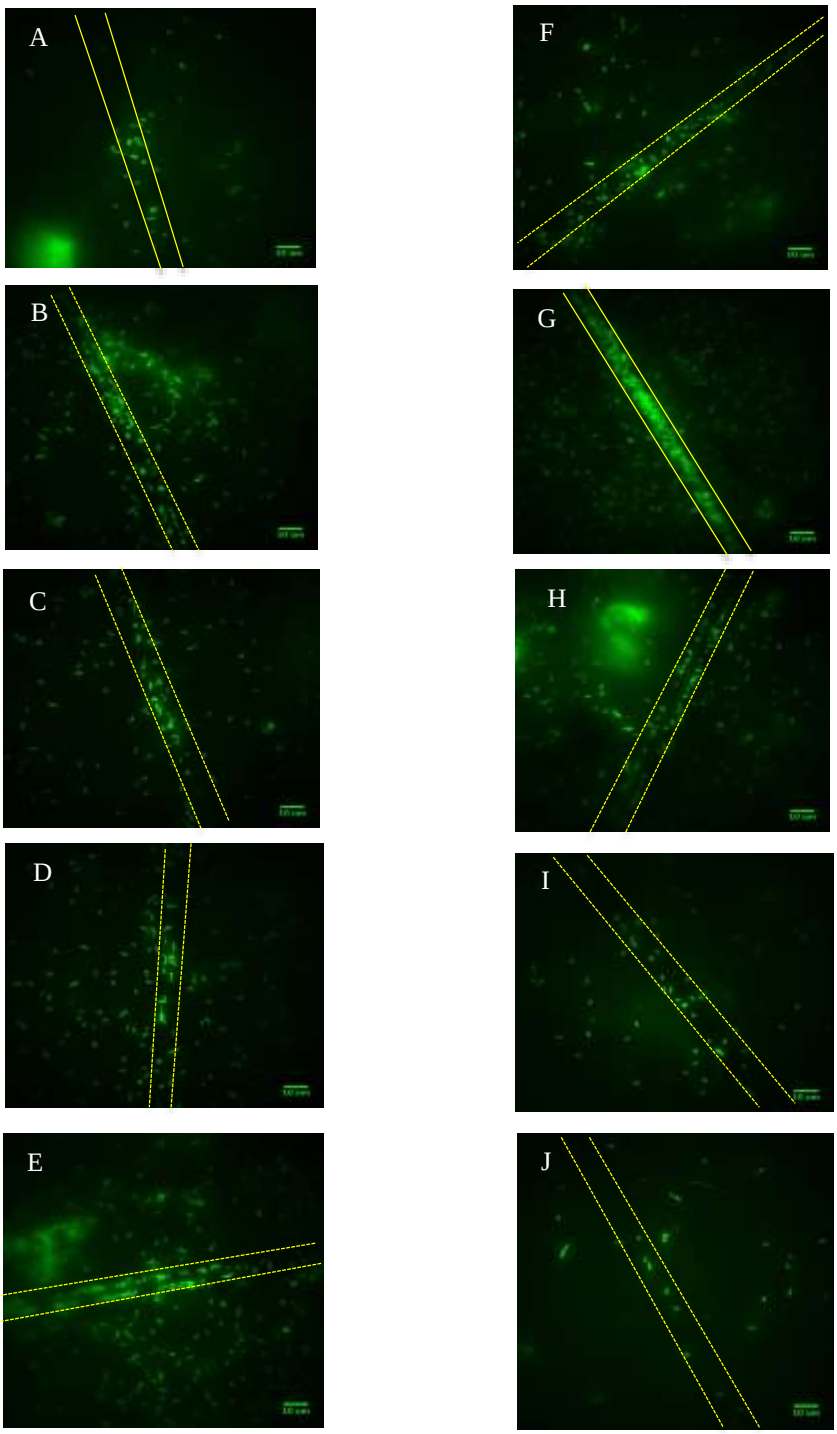


Figure 4.7 shows the development of an *A. butzleri* ED-1 (A-E) and an *Arcobacter* L (F-J) anodic biofilm over a time period of 120 h. Bacteria were tagged with GFP and anode fibre marked by yellow line. Scale bar represents 10 μm.

The cells attached to the anode were quantified using the Image J cell counter tool. The number of cells is given in Table 4.3.

Time (h)	Total cells	Total cells attached to the anode	Total cells unattached	% of cells attached to the anode
24	47.67 ± 3.21/ 160.33 ± 10.1	20.00 ± 5.29 /71.33 ± 1.53	27.67 ± 3.51 /89.00 ± 10.44	41.7 ± 8.92 /44.61 ± 3.0
48	145.67 ± 63.5/ 429.00 ± 40.15	85.00 ± 37.47 /235.33 ± 26.1	60.67 ± 29.50 /193.67 ± 14.67	59.28 ±8.63 /54.80 ± 1.13
72	117.33 ± 8.96 /143.33 ± 30. 27	52.00 ± 8.89 /47.33 ± 23.71	65.33 ± 11.02 /96.00 ± 18.73	44.4 ± 7.22 /31.92 ± 13.18
96	119.00 ± 8.96 /48.00 ± 9.64	41.00 ± 10.15 /18.67 ± 79.00	78 ± 12.29 /29.33 ± 6.11	34.54 ±9.12 /38.94 ± 2.55
120	295.67 ± 36.67 /37.33 ± 8.39	154.67 ±38.18 /13.67 ± 5.13	141 ± 25.16 /23.67 ± 8.5	52.02 ± 8.34 /37.15 ± 8.50

Table 4.3 shows the mean number of *A. butzleri* ED-1*Arcobacter* L in a 100 μm by 100 μm section of anodic biofilm on a 10 mm by 10 mm section of anode and the percentage of cells attached to the anode over a time period of 120 h. Cells were counted from a total of 30 images.

The redox potentials of the half-cells were measured every 24 h over the course of the experiment. Figure 4.8 compares the redox potentials generated by *A. butzleri* ED-1 and *Arcobacter* L.

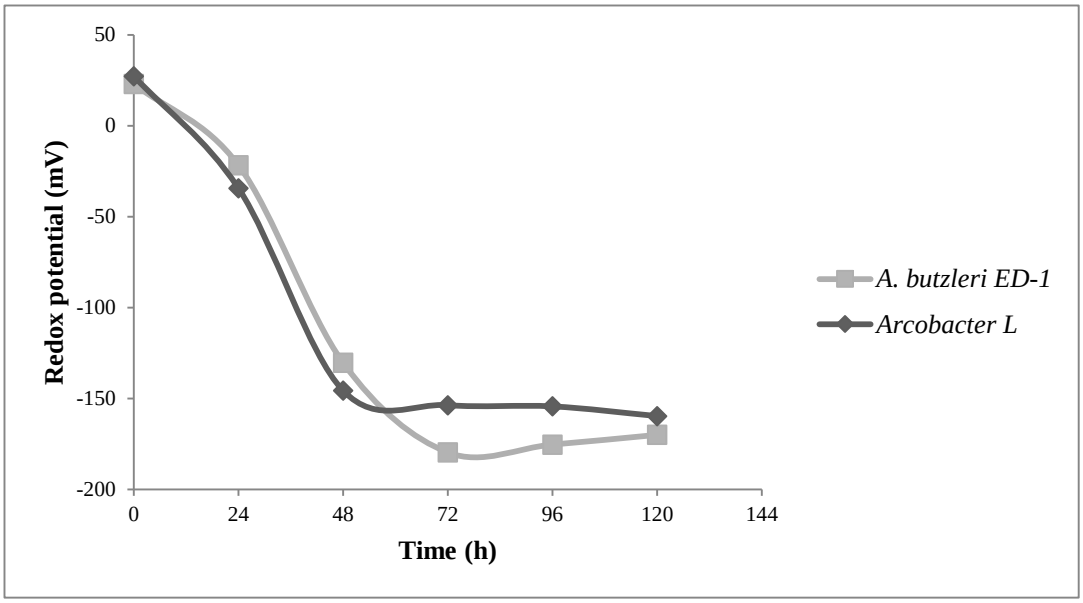


Figure 4.8 shows the mean redox potential generated by *A. butzleri* ED-1 and *Arcobacter* L over a period of 120 h. The redox potential of both cultures fell sharply in the first 48 h with a similar redox potential achieved for both *Arcobacter spp.* although the *Arcobacter* L redox potential falls faster than that of *A. butzleri* ED-1 which correlates with the higher number of cells on the anode (71.33/ μm^2 as opposed to 20. 00/ μm^2). After 48 h the *Arcobacter* L potential does not fall whereas the *A. butzleri* ED-1 redox potential continues to fall. This correlates with the detachment of *Arcobacter* L from the anode after 72 h. The redox potential is less negative than previously observed for *A. butzleri* ED-1 and there is less difference between the redox potential of *A. butzleri* ED-1 and *Arcobacter* L as previously observed (see chapter 3); this presumable due to oxygenation of the half MFC due to repeated sampling.

After 24 h the redox potential of both half cells fell from an initial potential of 25 mV and cells being to attach to the anode. More *Arcobacter* L cells attach to the anode (71.33 per 100 μm^2) then *A. butzleri* ED-1 (20 per 100 μm^2). This correlates with the more rapid decline in redox potential observed by *Arcobacter* L, although the percentage of both types of cell attached to the anode is similar at approximately

40%. The morphology of the biofilms reflects the differences in cell numbers with the *A. butzleri* ED-1 biofilm consisting of unevenly distributed cells, whereas the *Arcobacter* L is a much more homogenous if thin biofilm. In both sets of images approximately 60 % of all cells observed were not attached to the anode but were rather found free living in the interstitial fluid collected when the anode was harvested.

After 48 h the redox potential of both half-cells continued to fall with the redox potential of *Arcobacter* L half-cell once again being more negative than that of the *A. butzleri* ED-1 half-cell (-140 mV as opposed to -125 mV). At the 48 h time point both biofilms displayed the similar morphology of a homogenous but thin biofilm distributed across individual fibres of the anode with a number of cells inhabiting the interstitial fluid between the fibres with 59% of *A. butzleri* ED-1 being attached to the anode and 54% of *Arcobacter* L being attached to the anode. Once again the population of *Arcobacter* L was higher than that of ED-1 (429 and 125 cells per 100 μm^2 respectively), which correlates with the more negative redox potential observed for *Arcobacter* L.

After 72 h the redox potential of both half cells continued to decrease although the redox potential of the *A. butzleri* ED-1 half-cell dropped dramatically whereas the redox potential of the *Arcobacter* L half-cell decreased only slightly. The population reflects this with the number of *Arcobacter* L having dropped to 143.33 cells per $100\mu m^2$ and only a few cells scattered across the surface of the anode. The

population and morphology of the *A. butzleri* ED-1 anodic biofilm remained approximately the same.

After 96 h the redox potential of the *Arcobacter* L half-cell remains the same although the population of cells has further decreased to 48 cells/ 100 $\mu m^{2.}$ The redox potential of the *A. butzleri* ED-1 half-cell remains the same, as does the morphology of the biofilm although only 34% of cells are attached to the anode.

After 120 h the redox potential of the *A. butzleri* ED-1 half-cell increases although the population of cells rises to 295 per 100 μm^2 and the biofilm becomes more densely populated with 52% of cells being attached to the anode. The population of *Arcobacter* L decreases slightly to 37 cells per 100 μm^2 but the morphology and redox potential remain unchanged from 96 h.

4.3 Discussion

4.3.1 The genetic manipulation of *A. butzleri* ED-1 and *Arcobacter* L

The development of pJK1 and pMK1 and their introduction into *A. butzleri* ED-1 and *Arcobacter* L was important as it provided a valuable set of tools for the genetic manipulation of *Arcobacter spp*. pJK1/ pMK1 could be easily modified to create an *E. coli- Arcobacter* shuttle vector by removing the GFP/CFP cassette and replacing it with a multiple cloning site and could be used as an expression vector for a number of different genes. The triparental mating system provides a reliable method of

introducing exogenous DNA into *Arcobacter spp*. which allows for a variety of different studies to be performed including the introduction of suicide vectors for mutagenesis studies allowing for the functions of a variety of different genes in *Arcobacter spp*. to be tested. In the context of this project it allows the deletion of genes potentially involved in extracellular electron transfer in order to better. The current lack of available genetic tools and systems for *Arcobacter spp*. mean this work is important as it has increased the ease with which *Arcobacter spp.* can be genetically manipulated.

4.3.2 Biofilm formation of *A. butzleri* ED-1, *Arcobacter* L and mixed culture on glass coverslips

A pure culture of *Arcobacter* L progressed to the mature biofilm stage faster than *A. butzleri* ED-1. This was somewhat surprising as due to its association with the anode *A. butzleri* ED-1 was expected to have a greater predisposition towards biofilm formation (Fedorovich *et al*., 2009). In a mixed culture *Arcobacter* L was initially the more dominant species.

One possible explanation is differences in acetate utilisation by the two different strains. The metabolic reconstruction predicted that *Arcobacte*r L generates more energy carrying units from acetate than *A. butzleri* ED-1 (Ler 2009). *A. butzleri* ED-1 was predicted to yield 2$FADH_2$, 2NADH and 1 reduced ferredoxin from 3 molecules of acetate whereas *Arcobacter* L was predicted to yield 2$FADH_2$, NADH and 1 reduced Ferredoxin from 2 molecules of acetate (Ler 2009). Assuming the predicted

model of acetate utilisation is correct this would mean that overall *Arcobacter* L would generate more energy from the same amount of acetate provided and therefore grow at a faster rate. In order to confirm this experimentally *A. butzleri* ED-1 and *Arcobacter* L would need to be grown at a variety of acetate concentrations and the rate of growth compared; if this assumption is correct *Arcobacter* L will show a faster rate of growth no matter what the concentration of acetate. This could be investigated experimentally by comparing the growth of *A. butzleri* ED-1 and *Arcobacter* L at different concentrations of acetate; if the above assumption is correct *Arcobacter* L would have a final higher growth yield then *A. butzleri* ED-1 no matter what the concentration of acetate.

A further possible reason for the more rapid growth of *Arcobacter* L was that the AMM supplied every 24 h was not deoxygenated beforehand. *Arcobacter* L was predicted to be less suited to anaerobic respiration and more suited to aerobic respiration than *A. butzleri* ED-1, lacking several genes such as the genes for TorC (a TMAO redutase), biotin sulphoxide reductase and the nitrite reductase nrfNH (Ler 2009). Therefore an oxygenated medium may favour the initial growth of *Arcobacter* L meaning that it more quickly formed a mature biofilm. The supply of oxygenated AMM may also explain why *A. butzleri* ED-1 persisted as a biofilm for 96 h as opposed to 72 h. *Campylobacter spp*. shows an increased tendency for biofilm formation under aerobic conditions (Reuter *et al*., 2010). However this does not explain why *A. butzleri* ED-1 detached from the biofilm at the 120 h time point. Therefore the effect of oxygen on the *Arcobacter* L biofilm could be to promote more rapid growth and biofilm form formation and subsequently more rapid

detachment from the biofim. The effect of oxygenated medium on *A. butzleri* ED-1 and *Arcobacter* L biofilm formation could be tested by repeating the experiment using a supply of deoxygenated medium.

The faster growth rate of *Arcobacter* L due either to more efficient utilisation of acetate or it being better adapted to aerobic respiration, may also be responsible for the more rapid detachment of *Arcobacter* L from the biofilm. More rapid growth leads to a more rapid oxidation of acetate which results in an accumulation of H^+ thereby making pH more acidic. *C. jejuni* cells in biofilms have shown to lack an adaptive acid stress response (Murphy *et al*., 2006) and if *Arcobacter* L lacked a similar response acidification of the biofilm could promote detachment and dispersal of cells. If the assumption of a faster growth rate is correct pH becomes too acidic at 72 h for *Arcobacter* L to survive but *A. butzleri* ED-1 can survive due to a higher pH caused by slower growth (slower growth results in a lower concentration of H^+). It is also possible that *A. butzleri* ED-1 possesses acid stress responses that *Arcobacter* L does not, allowing it proliferate under more acidic conditions. In order to investigate this the experiment must be repeated at a range of different pH.

Depletion of nutrients was unlikely to be responsible for the detachment of cells from the biofilm as the cells were supplied with fresh medium every 24 h and nor is a depletion in oxygen tension likely to be responsible for biofilm dispersion because, as previously discussed the AMM supplied was not deoxygenated.

When visualised in a mature biofilm state (72 h for *Arcobacter* L and 96 h for *A. butzleri* ED-1) a small percentage (8.3%) of *Arcobacter* L cells display an altered morphology (shorter and rounder looking than other *Arcobacter* L and more fluorescent) whereas *A. butzleri* ED-1 cells do not. *Campylobacter spp*. have been show to adopt a cocci like morphology in the stationary phase of growth (Kelly *et al*., 2001) particularly in the centre of the colony (Ng *et al*., 1985). Therefore it is likely that the altered morphology *Arcobacter* L are cells that simply underwent degenerative change (Ng *et al*., 1985), which is observed in cells in *Campylobacter* cultures after 72 h or more of growth (Kelly *et al*., 2001). The purity of the culture was confirmed by light microscopy and plating of the planktonic phase onto Houf blood agar (Houf *et al*., 2001) (see chapter 2 for composition) therefore the cells with altered morphology were determined not to be contaminants.

A mixed *Arcobacter spp*. biofilm showed a more rapid progression to mature biofilm stage (approximately 48 h) and stably remained in the mature biofilm state until 120-h. For the first 96 h of growth *Arcobacter* L was the dominant species, which was presumably due to the bacterium's ability to more effectively use acetate as a carbon source or due to oxygenated medium favouring *Arcobacter* L growth. The more rapid development of the biofilm, greater cellular numbers and persistence of the mature biofilm suggest that there is synergistic relationship between the two species allowing them to grow more efficiently and better withstand stresses such as oxygen deprivation and acid stress which promotes detachment as discussed above. However the precise details of this synergistic relationship remains an open question.

Therefore it was concluded that this particular culture of *Arcobacter* L progressed to a mature biofilm more rapidly than *A. butzleri* ED-1 and this was possibly due to *Arcobacter* L more effectively using acetate as a carbon source or oxygenated AMM favouring *Arcobacter* L growth i.e. *Arocbacter* L is more adapted to aerobic growth than *A. butzleri* ED-1. It was also concluded that this particular culture of *A. butzleri* ED-1 maintained a more stable biofilm than *Arcobacter* L which was possibly due to aerobic conditions promoting *A. butzleri* ED-1 biofilm formation or *A. butzleri* ED-1 being more resistant to acidity than *Arcobacter* L. From the above results it was concluded that *A. butzleri* ED-1 and *Arcobacter* L possibly display a synergistic relationship when grown in co-culture.

However it must be noted that these conclusions were drawn from a single set of cultures and therefore the results are highly subject to experimental error and as such the experiment must be repeated in triplicate before more detailed conclusions about *Arcobacter spp*. biofilm formation can be drawn.

4.3.3 Anodic biofilm formation by *A. butzleri* ED-1 and *Arcobacter* L

When cultured in a half MFC *Arcobacter* L initially formed a more mature biofilm than *A. butzleri* ED-1 in a time frame similar to that observed for the experiments on glass coverslips. As observed on the glass coverslips *Arcobacter* L detached from the biofilm after a shorter length of time (72 h) than *A. butzleri* ED-1 which formed a

more stable biofilm that persisted for 120 h. This was unexpected due to *Arcobacter* L being found in association with the planktonic phase of the MFC (Fedorovich *et al*., 2009) and subsequent genomic analysis predicting *Arcobacter* L to be more suited to aerobic respiration than *A. butzleri* ED-1 lacking proteins such as TorC (a TMAO redutase), biotin sulphoxide reductase and the nitrite reductase nrfNH (Ler 2009) although it must be noted that due to the electrogenic mechanisms of *Arcobacter spp*. the importance of these proteins in electrogenesis is unknown.

As discussed in section 4.3.2 the differences in the bacteria's suitability to aerobic and anaerobic respiration and the levels of oxygen in the AMM to the half MFCs could explain the differences in anodic biofilm observation. *Arcobacter* L is thought to be more suited to aerobic respiration and *A. butzleri* ED-1 was thought to be more suited to anaerobic respiration with genomic analysis showing *A. butzleri* ED-1 possessing several genes for anaerobic respiration that *Arcobacter* L lacks. *A. butzleri* ED-1 was found to encode TorC (a TMAO redutase), biotin sulphoxide reductase and the nitrite reductase nrfNH (Ler 2009), all of which are used in anaerobic respiration. The medium supplied to the half MFC was not deoxygenated beforehand therefore the more oxygenated medium could initially favour the growth of *Arcobacter* L and subsequent biofilm formation. As discussed above more rapid growth could lead to more rapid acidification of the biofilm, which could promote detachment. The depletion of oxygen in the half MFC over time could also force the more aerobic *Arcobacter* L to detach from the biofilm, whereas the *A. butzleri* ED-1 biofilm could proliferate within the anodic environment. This does not explain the rise in redox potential and increase in *A. butzleri* ED-1 numbers in the anodic biofilm

after 120 h. One possible explanation for this is the increase of oxygen in the system as it was opened multiple times for sampling of the anode. As previously discussed *Campylobacter spp*. show increased biofilm formation under aerobic conditions (Reuter *et al*., 2010) and therefore increased oxygenation could force *A. butzleri* ED-1 in the planktonic phase to form a biofilm. This would explain why an increase in the number of cells in the *A. butzleri* ED-1 anodic biofilm did not result in a fall (i.e. becoming more negative) of the redox potential. In order to test this the experiment should be repeated under more stringent anaerobic conditions such as those used to culture *A. butzleri* ED-1 for iTRAQ analysis (see chapter 2 for culture conditions). Alternatively a system that does not require sampling for imaging should be used akin to the MFC used by Franks *et al*. i.e. a small MFC used directly for imaging.

As previously discussed in section 4.3.2 the more rapid growth of *Arcobacter* L could lead to acidification of the biofilm, which would promote the dispersal of cells and *A. butezleri* ED-1 may be more resistant to acid stress than *Arcobacter* L. This theory could be tested by cultivating anodic biofilms at different pH to investigate whether this effects biofilm formation by either species.

The half MFC set-ups used in this experiment were not supplied with fresh AMM and therefore nutrient deprivation could also have resulted in detachment of *Arcobacter* L from the anode, especially since *Arcobacter* L grew faster than *A. butzleri* ED-1 meaning there would be less nutrients available for growth at later time points. This however is unlikely considering similar dispersal of *Arcobacter* L was observed on the glass coverslip biofilms which were supplied with fresh AMM

every 24 h. So while nutrient deprivation is an unlikely reason for the detachment of *Arcobacter* L the experiments should nevertheless be repeated with regular feeding as per the experiments discussed in section 4.3.2.

A. butzleri ED-1 and *Arcobacter* L both form thin anodic biofilms of only a few cells thick. This suggests that the bacteria may employ a different electrogenic mechanism then *Geobacter sulfurrreducens*, which forms a thick anodic biofilm of approximately 50μm (Nevin *et al*., 2009). It must be noted that this takes approximately 400 h and so an accurate comparison cannot be made without performing the same experiments on *Arcobacter spp*. for an equivalent time period. It must also be noted that without repeats of the above experiments under more stringent conditions the idea of a different method for electrogenesis by *Arcobacter spp*. is highly speculative.

Based on these results it can therefore be concluded that there the difference between *A. butzleri* ED-1 and *Arcobacter* L anodic biofilms in half MFCs is that *A. butzleri* ED-1 is able to maintain a more stable biofilm over a longer period of time than *Arcobacter* L and this may explain why cultures of A. *butzleri* ED-1 tend to produce more negative redox potentials than *Arcobacter* L. However the experiments need to be repeated under more stringent anaerobic conditions and with regular feeding and in any case must be repeated as these results were drawn from a single set of half MFC cultures.

4.3.4 A possible new model for electrogenesis by *A. butzleri* ED-1 and *Arcobacter* L?

There was also a high number of planktonic cells observed in the half MFC used for these experiments as well as in previous half MFC cultures of *A. butzleri* ED-1 and *Arcobacter* L (see chapter 3 and chapter 5). This coupled with the thin anodic biofilms observed for both *A. butzleri* ED-1 and *Arcobacter* L suggest that *Arcobacter spp*. generate electricity by a new model of electrogenesis involving planktonic cells.

One possibility is that *A. butzleri* ED-1 and *Arcobacter* L generate electricity through both the use of mediator compounds produced by the planktonic phase cells and by direct contact pathways of extracellular electron transfer. *Shewanella oneidensis* has also been shown to produce electricity by direct contact mechanisms and mediator compounds working in tandem, with direct contact mechanisms and mediators transporting electrons throughout the anodic biofilm (Marsili *et al*., 2008) and mediator compounds transferring electrons from planktonic phase bacteria to the anode (Lanthier *et al*., 2008). This is discussed in more detail in chapter 1. Therefore it is possible that *Arcobacter spp*. utilise the anode in a similar fashion, although the lack of nanowires means that *Arcobacter spp.* can only produce an anodic biofilm a few cells thick.

It must be noted that initial studies by cyclic voltammetry suggested that neither *A. butzleri* ED-1 nor *Arcobacter* L produced mediator compounds (Fedorovich unpublished data); however since *Arcobacter* have now been cultured in pure culture using a more suitable medium these studies must be repeated in order to confirm this result.

This model is highly speculative and based on data taken from a single set of half MFC experiments plus other experiments. In order to develop it further the experiments must be repeated and performed under more stringent conditions as discussed above.

4.4 Conclusions

The following conclusions can be drawn from these experiments.

Firstly triparental mating using RK2 *oriT* and the pRK600 helper plasmid is a suitable method for introducing exogenous DNA into *Arcobacter spp*. This provides an invaluable tool for the further genetic manipulation of *Arcobacter spp*.

From these particular cultures it can be concluded that *Arcobacter* L initially forms a mature biofilm more rapidly than *A. butzleri* ED-1 but the *A. butzleri* ED-1 biofilm is maintained for a longer period of time. This may explain why *A. butzleri* ED-1 cultures are generally more electrogenic than *Arcobacter* L cultures although the

experiments need to be repeated as detailed in sections 4.3.3 and 4.3.4 as the reasons behind this remain largely unknown and mostly speculative.

The results obtained from this experiment also suggest a new model for electrogenesis by *Arcobacter spp*. involving both direct contact and mediators as a means of extracellular transportation. However, more experimentation is needed for this model to be developed in detail.

In the mixed culture studied there was a possible symbiotic relationship between *A. butzleri* ED-1 and *Arcobacter* L; however this was based on a single mixed culture and thus the experiment must be repeated as detailed in section 4.3.3.

In summary the main conclusions drawn from this set of experiments is that the main difference between *A. butzleri* ED-1 and *Arcobacter* L biofilm formation is that the *Arcobacter* L biofilm forms and disperses more rapidly than the *A. butzleri* ED-1.The second conclusion is that triparental mating is a valid method for introducing exogenous DNA into *Arcobacter spp*.

Chapter 5

Comparative, quantitative proteomic analysis of *A. butzleri* ED-1 harvested from a half MFC anode, the planktonic phase of a half MFC and an aerobic planktonic culture

(Performed in collaboration with Dr. Ana G. Pereira Medrano, University of Sheffield)

5.1 Introduction

A. butzleri ED-1 and *Arcobacter* L were the first ε proteobacterium found to be capable of electrogenesis. *Arcobacter spp*. has subsequently been isolated from other MFC systems; *Arcobacter nitrofigilis* were isolated from plankton fed MFC, where at peak power of the system they represented 2% of the total population. (White *et al*., 2009). A strain of *Arcobacter* closely related to *A. butzleri* ED-1 was isolated from a MFC inoculated with activated sludge (Nien *et al*., 2011). This suggests that *Arcobacter spp*. are electrogenic although their mechanisms of electrogenesis remain unknown.

The most well characterised pathways of extracellular electron transfer are found in *Geobacter sulfurreducens*; therefore the first step in elucidating the pathways of extracellular electron transfer of *A. butzleri* ED-1 is to use the genomic data and see if there are any genes/ proteins analogous to those involved in extracellular electron transfer in *Geobacter sulfurreducens.*

Geobacter sulfurreducens cells form a thick conductive biofilm (i.e. a biofilm with an electrically conductive extracellular matrix) of 50 μm or more (Franks *et al.*, 2008) due to their ability to produce nano-wires, electrically conductive pili that facilitate the transfer of electrons across long distances (Regurea *et al*., 2005). The electrogenic process in *Geobacter sulfurreducens* is dependent on two c type cytochromes OmcB and OmcZ, which are found to be prevalent in the outer membrane and extracellular matrix of anodic biofilms by a transcriptomic study on

anode associated *Geobacter sulfurreducens* (Nevin *et al.*, 2009). BLAST analysis of OmcB, OmcZ and PilA show that there are no proteins of significant homology within the *A. butzleri* ED-1 genome.

The lack of OmcB, OmcZ and PilA analogues and the thin anodic biofilm (see chapter 4) formed by *A. butzleri* ED-1 suggests that the bacteria's pathways of extracellular electron transfer may be distinct from those of *Geobacter sulfurreducens* although it is equally possible that different proteins perform similar roles in *A. butzleri* ED-1.. It was decided to perform a "whole cell" study on *A. butzleri* ED-1 to determine what proteins are potentially involved in the electrogenic process by *A. butzleri* ED-1.

5.1.1 Methods of studying the extracellular electron transfer pathways of *A. butzleri* ED-1

There are three main categories of method for performing a "whole cell" study of the extracellular electron pathways of *A. butzleri* ED-1 a genomic approach, transcriptomic approach and a proteomic approach.

The genomic approach would be centred on a mutagenesis method such as transposon mutagenesis. It is possible to introduce exogenous DNA into *Arcobacter spp*. by triparental mating (see chapter 4, Table 4.1) and would be the cheapest method to employ. However the lack of an effective screen for electrogenesis/ metal

reduction (see section 3.8.1) meant it would be an impractical method to employ, as mutants with diminished electrogenic ability could not be easily detected.

A transcriptomic approach i.e. the application of microarrays has been used to great success to identify the electrogenic mechanisms in *Geobacter sulfurreducens* (Nevin *et al.*, 2009), and as such was, in theory, the most suitable method to perform a similar study on *A. butzleri* ED-1 anodic biofilms. However a transcriptomic approach would require the construction of bacterium specific microarrays and specialist equipment, which was not within available resources.

A proteomic approach has not been used for the study of electrogenesis before; although it has been used to study bacteria under a variety of other conditions (see below). This approach is only moderately expensive and could be feasibly carried out with suitable collaboration.

Based on the above factors it was decided to employ a proteomic approach to study the electrogenic mechanism and anodic physiology of *A. butzleri* ED-1.

5.1.2 The use of shotgun proteomics to study bacteria

The use of shotgun proteomics is novel in terms of the study of extracellular electron transfer pathways; however it has been employed to study bacteria under a variety of other conditions. Shotgun proteomics have been used to study nitrogen fixation in

the cyanobacterium *Anabaena variabilis* ATCC 29413 (Barrios-Llerena *et al*., 2006), cross-strain variation based on amino acid divergence (Denef *et al*., 2007) and the study of protein expression in subgingival biofilms (Pham *et al*., 2010). This study was used to simultaneously identify and quantify changes in protein expression in the anaerobic bacteria *Tanerrella forsythia* when cultured as a sub gingival biofilm (Pham *et al*., 2010). The study found an up-regulation in putative outer membrane proteins associated with biofilm formation, the down- regulation of proteins associated with the butyrate production pathway and the up-regulation of putative oxidative stress proteins (Pham *et al*., 2010). This study showed a number of physiological changes that may explain the persistence and immune evasion of the bacteria in the oral cavity (Pham *et al*., 2010).

The use of proteomics to study biofilm protein expression is of particular relevance to the study of *A. butzleri* ED-1 on the anode as it shows that the proteomic approach is suitable for studying a biofilm.

5.1.2.1 The proteomics of biofilm formation

While there has been no proteomic study of biofilm formation by *Arcobacter spp*. There have been numerous proteomic studies of biofilm formation by a wide range of other species including the closely related *Campylobacter spp*.

Proteomic analysis of *Campylobacter jejuni* grown as a biofilm on a nitrocellulose membrane showed that proteins of the motility complex were up-regulated compared to planktonic phase bacteria (Kalmokoff *et al*., 2006). These proteins included the flagellins FlaA and FlaB, proteins of the basal body FlgB and FlgE and the chemotactic factor CheA (Kalmokoff *et al*., 2006). In this particular study two known adhesins Peb1 and FlaC were also found to be up-regulated (Kalmokoff *et al*., 2006). Other studies have shown that flagellins are up-regulated in *Campylobacter* biofilm formation and that mutants lacking the genes for flagellin are less able to form biofilms (Joshua *et al*., 2006). In addition to adhesins and flagella a number of other proteins have been found to be involved in biofilm formation in *Campylobacter spp*. For example in *C. jejuni* the stress response proteins groEL and groES were found to be up-regulated during biofilm formation as were the oxidative stress responses Tpx and Ahp (Kalmokoff *et al*., 2006). More recent studies have shown that the cprS sensor kinase has been found to be important in regulating *C. jejuni* biofilm formation including the FlaA production described above (Svensson *et al*., 2009).

Proteomic analysis of other bacteria have also shown that a variety of different proteins are involved in biofilm formation. *Bacillus cereus* was found to display up-regulation of the σ^{54} regulator protein yhbH during biofilm formation, which is thought to play a central role in the switch between planktonic growth to biofilm growth by the bacterium (Oosthuizen *et al*., 2002). Flagella have also been found to be important in *Bacillus cereus* biofilm formation with flagella deletion mutants displaying decreased ability to form biofilms (Houry *et al*., 2010). Proteomic analysis of *Streptococcus mutans* LuxS mediated sensing was found to be important in biofilm formation (Wen and Burne 2004) and in *Psuedomonas aeruginosa* the retS sensor kinase/ response regulator hybrid is critical to the expression of the type III secretion system required for EPS production and subsequent biofilm formation (Goodman *et al*., 2004).

The above studies show that biofilm formation is a complex process involving a wide variety of different types of proteins including adhesins and a range of regulatory and sensor proteins.

While there has been no proteomic studies of anodic biofilms there have been transcriptomic studies which show proteins up-regulated in an anodic biofilm. As discussed previously *Geobacter sulfurreducens* has displayed several up-regulated proteins when taken from the anode of an MFC, these include the outer membrane cytochrome OmcZ and the pillin protein PilA (Nevin *et al*., 2009). This is covered in more detail in chapter 1. PilA is of particular interest as it had been shown to be uninvolved in biofilm formation with PilA deletion mutants being able to form

biofilms but unable to reduce Fe^{3+} or generate electrical current in a MFC (Reguera *et al*., 2005, Regurea *et al*., 2006). It was therefore concluded that PilA is not important in biofilm formation by *Geobacter sulfurreducens* but in extracellular electron transfer instead.

Therefore it might be expected to observe up-regulated adhesins, flagellins, sensor and regulatory proteins and various stress response proteins in *A. butzleri* ED-1 harvested from the anode. If the bacterium's electrogenic mechanisms are similar to those of *Geobacter sulfurreducens* it may be expected to observe up-regulated electron transport proteins.

5.1.3 Different method of shotgun proteomics

There are a number of different shotgun proteomic methods that could conceivably be used for a comparative proteomic analysis of *A. butzleri* ED-1. Shotgun proteomics is preferable to the more traditional approach of 2D gel electrophoresis as it allows for rapid comparison and quantification of different samples, does not show bias against membrane proteins and does not have overlapping samples which make identification by mass spectrometry difficult (Wu and MaCoss 2002). There are several commonly used methods of quantitative shotgun proteomics (Ong and Mann 2005).

Ion current (XIC) based comparison is perhaps the simplest method comparing the intensity of peptides between two or more different states (Ong and Mann 2005). The

main advantage is that it does not require any labelling or modification and so can be performed with any type of sample. The main disadvantage of this method is that it is quite easy to obtain different intensities of the same peptide between different runs (Ong and Mann 2005). In short XIC based comparison serves as a "rough and ready" form of protein quantification (Ong and Mann 2005).

The current "gold standard" method of quantitative proteomics is stable isotope labelling of samples (Ong and Mann 2005). There are four main methods to go about this 1) spiking in an isotopically labelled analogue 2) tagging a protein with a chemical, isotopic tag 3) culture cells so they incorporate the label metabolically 4) chemically tagging the protein during protein digestion (Ong and Mann 2005). It was decided to use a chemical tagging method.

In the chemical tagging approach stable chemical isotopes are attached to reactive sites on the peptide such as amine groups, or sulfhydryl groups (Ong and Mann 2005). Chemically tagging has the same advantages as isotope based tagging, in addition to not having to work with radioactive compounds (Ong and Mann 2005). A potential disadvantage is that the chemical tag only attaches to peptides containing the correct reactive group (Ong and Mann 2005) although this is not a problem if the chemical tag reacts with amine groups (Ong and Mann 2005). There are many different available chemical-labelling systems; one such system is isobaric tags for relative and absolute quantification (iTRAQ).

iTRAQ uses a set of isobaric reagents that attach to the amine groups of peptides. iTRAQ is a straightforward technique to perform and has been successfully used to perform proteomic studies of prokaryotes and eukaryotes (Medrano *et al*., 2013) and therefore was chosen to study *A. butzleri* ED-1 in a half MFC environment. The iTRAQ technique is discussed further below.

5.1.4 iTRAQ

The iTRAQ technique is a shotgun proteomic technique based around tagging peptides with isobaric reagents (i.e. reagents with the same molecular mass) that allow for simultaneous identification and quantification of peptides (Aggarwal *et al*, 2006) and attach to the N terminus and amine groups of the peptides (Wiese *et al*., 2007). The isobaric mass design of the labels means that label proteins are not any different in mass and so their corresponding proteolytic peptides appear as single peaks in MS scans (Wiese *et al*., 2007). This coupled with the multiplex nature of iTRAQ and the high collision energy needed to liberate the labels from the peptide means that iTRAQ produces highly accurate results (Wiese *et al*., 2007). The labelled proteins are digested separated by LC chromatography based on hydrophobicity before being analysed by tandem MS-MS where the first MS quantifies the number of fragments and the second MS scan is used to identify the most abundant peaks (Wiese *et al*., 2007). Figure 5.1 illustrates the basic process of the iTRAQ technique.

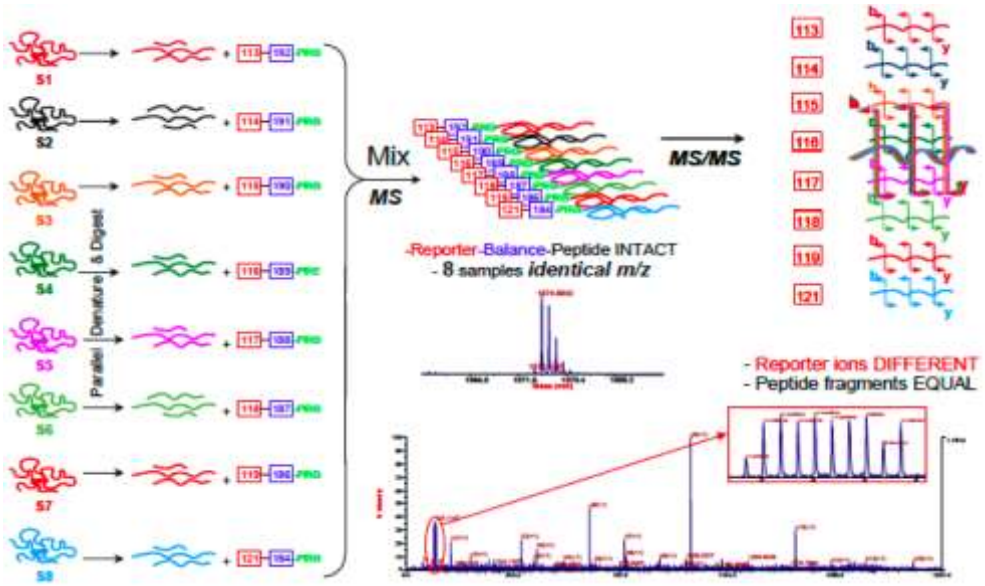


Figure 5. 1 shows the process of iTRAQ. Figure 5.1 supplied by Medrano *et al*. (personal communication). The samples are denatured and digested then tagged with isobaric residues at the N terminal. They are then separated chromatography using a strong cation exchange column The samples are then subject to reverse phase liquid chromatography coupled to tandem mass spectrometry (MS) .The first MS quantifies the peptide fragments then the second MS is then used to identify the most abundant fragments from the first round of MS. The data is then subject to intensive computer analysis by software such as Compass Data analysis and Analyst QS (Ow *et al.*, 2007).

As Figure 5.1 states iTRAQ can be performed in both 4 plex and 8 plex reactions which means that multiple samples can be simultaneously analysed and compared; making it ideally suited for the 3 way comparison used in this study.

5.1. 5 Aims and objectives

The aim of this study were to compare the proteomes of *A. butzleri* ED-1 harvested from the anode, the planktonic phase of a half MFC and an aerobic planktonic culture.

A proteomic comparison would show what proteins were up-regulated at the anode and therefore potentially important in electrogenesis. If the electrogenic mechanisms of *A. butzleri* ED-1 were similar to those of *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 than it would be expected to see electron transporter proteins and adhesins up-regulated in cells harvested from the anode compared to cells harvested from the planktonic phase. If the mechanisms of electrogenesis are different than a different types of protein may be observed. Based on the results from biofilm imaging (see chapter 4) it is possible that *A. butzleri* ED-1 performs electrogenesis by a mechanism distinct from the better characterised mechanisms of *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1. At this stage the idea of a novel electrogenic mechanism for *A.butzleri* ED-1 is highly speculative and proteomic analysis would possibly elucidate this further and provide insights into what proteins are involved in electrogenesis by the bacterium.

Therefore in summation the aims of the iTRAQ analysis are twofold

1. To show what proteins are up-regulated at the anode and therefore have a potential role in electrogenesis. These proteins can then be used as the target for deletions mutants in order to better understand their role in the electrogenic mechanisms of *A. butzleri* ED-1.
2. To see whether adhesisns and electron transporter proteins are up-regulated at the anode thus showing whether the electrogenic mechanisms of *A. butzleri* ED-1 are similar or distinct from those of *Geobacter sulffureducens* and *Shewanella oneidensis* MR-1. If similar it would be expected to observe an up-regulation of adhesins and electron transporter proteins in *A. butzleri* ED-1 harvested from the anode.

5.2 Results

5.2.1 The use of redox indicators to develop a screen for electrogenesis

The ability of *A. butzleri* ED-1 to reduce a variety of redox indicators and metal oxides, in order to screen for electrogenic ability was investigated. This screen was to be used to detect mutants created by transposon mutagenesis that were deficient in electrogenic ability. Transposon mutagenesis was considered as an alternate method to iTRAQ for studying the electrogenic mechanism of *A. butzleri* ED-1.

Solid and liquid cultures of *A. butzleri* ED-1 containing either redox indicator or metal oxide were incubated under anaerobic conditions alongside *E. coli* which

served as a negative control. The redox indicators and metal oxides used are listed in Table 5.1.

Redox compound	E° (V)	Oxidised state colour	Reduced state colour
Methylene blue	11	Blue	colourless
Nile Blue	-142	Blue	colourless
Indigocarmine	-130	Blue	yellow
Indigo	-400	Blue	yellow
Neutral red	-325	Red	colourless
Ferric oxyhydroxide	-200	Brick Red	Blue/Black
Ferric citrate	-200	Brick Red	Blue/ Black

Table 5.1 lists the redox compounds tested as a potential screen for electrogenesis and gives their E°V i.e. the voltage at which the compound changes from an oxidised to reduced state. These compounds all produce distinct colour changes when they change from oxidised to reduced state, which theoretically made them ideal for a colorimetric assessment of the electrogenic ability of individual compounds.

Colour change was observed for *A. butzleri* ED-1 and *E. coli* when grown on methylene blue in both liquid culture and agar plates. *A. butzleri* ED-1 but not *E. coli* produced a colour change when grown on Nile blue and Indigo carmine; although on the agar plates the colour change was not limited to a single colony.

No colour change was observed for either *A. butzleri* ED-1 or *E. coli* when cultured in the presence of Neutral Red, Ferric oxyhydroxide, ferric citrate or Indigo. Ferric oxyhydroxide and ferric citrate were retested with a small amount of ferrozine added to the medium, as ferrozine turns purple in the presence of Fe^{2+} (Stookey 1970). Either no colour change was observed after incubation or in the case of *E. coli* cultures the medium turned purple instantly due to Fe^{2+} already in the medium.

5.3.2The preparation of samples for iTRAQ analysis

A half MFC set up was used to prepare *A. butzleri* ED-1 under MFC like conditions for iTRAQ analysis (see Chapter 2, section 2.2.1). To ensure the cells were representative of an anaerobic, MFC-like environment the cells were grown under anaerobic conditions (see Chapter 2, section 2.2.1). To produce sufficient quantities of protein the cells were grown for 300 h and subjected to feeding every 24 h after the first 96 h of growth. The feeding regime consisted of removing 25 mL of culture and replacing with 25 mL of fresh AMM (with 30 mM acetate serving as the sole carbon source, pH adjusted to 6.5). *A. butzleri* ED-1 was also grown under aerobic conditions for 72 h. The redox potentials and optical densities of the cells prepared under anaerobic conditions are shown in Figure 5. 2. Tables 5.2 and 5. 3 detail the amounts of protein per sample for anaerobic planktonic and anode associated cells respectively.

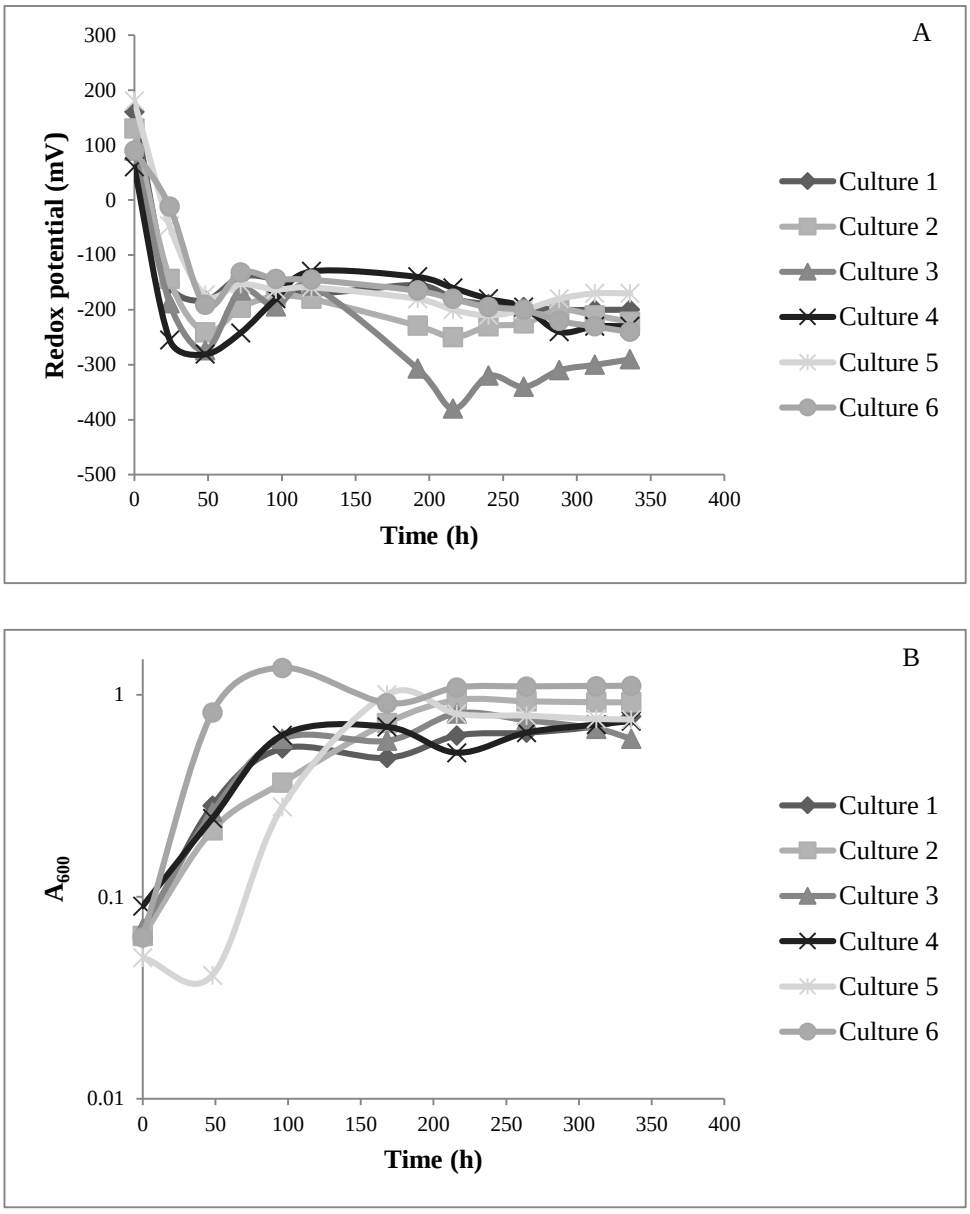


Figure 5. 2 show the redox potentials (A) and growth (B) of the half-cell cultures used for iTRAQ analysis over a 336 h time period. Two sets of three half-cell cultures were prepared i.e. one set of three was grown and harvested and then the second set. All half cells reached a redox potential of at least -200 mV within 48 h of growth, which was quicker than previously observed, due to the deoxygenated nature of the basal medium. Final A_{600} was between 0.8 and 1.0 which was again higher than observed and due to the intensive feeding regime used to increase cell yields in the half MFC cultures.

Culture	Absorbance (600nm)	Pellet Weight (g)	Protein (mg)	Potential (mV)
1	0.783	0.5	17.2	-200
2	0.920	0.47	18.0	-222
3	0.606	0.44	16.4	-290
4	0.741	0.57	14.4	-230
5	0.756	0.28	16.8	-170
6	1.109	0.57	16.4	-240
Mean	0.819 ± 0.174	0.47 ± 0.11	16.5 ±1.2	-225.3 ± 40.33

Table 5 2 shows the final absorbance, pellet weight amounts of protein and redox potential of planktonic phase of six half-cell cultures after 336 h of growth. Pellet weight is the "wet weight" of the cell pellet harvested from 200 mL of culture by centrifugation. The amount of protein was quantified in a 1mL sample of culture using the Bradford reagent and extrapolated for 200 mL.

Culture	Absorbance (600nm)	Pellet Weight (g)	Protein (mg)	Potential (mV)
1	0.102	0.11	3.2	-200
2	0.090	0.16	3.2	-222
3	0.058	0.19	3.2	-290
4	0.107	0.10	3.6	-230
5	0.099	0.16	3.2	-170
6	0.104	0.17	3.2	-240
Mean	0.093 ± 0.018	0.15 ± 0.04	3.3 ± 0.2	-225.3 ± 40.33

Table 5. 3 shows the final absorbance, pellet weight amounts of protein and redox potential of the anodic phase of six half cell cultures after 336 h of growth. Pellet weight is the "wet weight" of the cell pellet harvested from 100 mL of dislodged cells by centrifugation. The amount of protein was quantified in a 1 mL sample of dislodged cells using the Bradford reagent and extrapolated for 100 mL.

The cultures of *A.butzleri* ED-1 reached a highly negative redox potential (mean -225.3 ±40.3) after 336 h of growth The redox potentials showed a rapid decrease within the first 50 h of growth and then rose slightly reaching their final redox potentials with the exception of culture 3, which exhibited a further drop in redox potential. Light microscopy confirmed that the cultures were pure and that this variation was not due to contamination.

The growth of the cells was high (mean 0.819±0.173); which was higher than previously observed for cultures *A. butzleri* ED-1 prepared under half MFC conditions (see chapter 3). Like previous half MFC cultures there was a high number of cells in the planktonic phase of the six cultures.

The amount of protein in anaerobic planktonic samples was approximately 5* higher than in samples taken from the anode, this correlates with the higher number of cells in the planktonic phase.

5.2.3 iTRAQ analysis of samples (work performed by Dr. Ana G. Pereira Medrano *et al*.)

Protein was harvested from the cell pellet by the application of ultra-sonication, liquid nitrogen based cracking and water bath based sonication. 100 μg of protein (for comparison of aerobic planktonic cells to anaerobic planktonic cells) or 50 μg of protein (for comparison of aerobic planktonic to anode associated cells) was then taken and analysed via iTRAQ.

The samples were labelled with iTRAQ reagents according to the protocol given by Pham *et al*. The samples were then denatured with sodium deoxycolate (if insoluble) before being digested with a mix of trypsin and chymotrypsin. The samples were then subject to chromatography and mass spectrometry as per Pham *et al*. using both a UHR-QToF and a QStarXL. A more complete description of the process is given in Figure 5.1.

The raw data obtained from the tandem MS-MS was analysed and converted into a usable form by the use of Compass Data Analysis v. 4.02 (Bruker Daltonics),if

generated from a UHR-QToF, or mascot.dll embedded script (V1.6) coupled with Analyst QS 1.1.1 (Applied Biosystems), If generated from the QstarXL. The data from both different types of MS were integrated using the Phenyx algorithm (Colinge *et al.*, 2003). The resultant data was then integrated with the *A. butzleri* ED-1 genome (http://www.ebi.ac.uk/ena/data/view/AP012047 2011). The combined data sets were then used to generate Tables 5. 4 and 5.5.

The tables show the differences in proteins between anode - associated cells and aerobic planktonic cells. Flagellin (ABED_2065) was up regulated 2.4 fold at the anode. The proteins methylcitrate synthase (ABED_0260, 1.8-fold), bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase (ABED_1343, 1.6-fold), and 2-methylisocitrate lyase (ABED_0259, 3.4 fold) were all up regulated at the anode. These are all proteins of the methylcitrate cycle, which suggests this cycle is important in metabolism at the anode.

No electron transport proteins were up regulated at the anode with cytochromes c oxidase bb3 subunit II (ABED_1884) and subunit III (ABED_1886) being down regulated 2.1 and 3.1 fold respectively. Fumarate reductase iron sulfur subunit (ABED_0282) and flavoprotein subunit (ABED_0283) were down regulated 1.2 and 1.3 fold respectively. There was no detectable change in the levels of any other electron transport proteins expressed at the anode.

No other adhesins proteins with the exception of flagellin were up-regulated at the anode with fibronectin binding protein (ABED_0457) being down regulated 1.4 fold. There was no change in the levels of any other adhesins at the anode.

iTRAQ	Locus Tag	Protein Description	Functional category[a]	Loc.[b]	Fold change Anaerobic planktonic *vs* aerobic planktonic[c]	No. Peptides	Score[d]	*p*-value
1	ABED_0345	30S ribosomal protein S15	TL	CYT	-3.8	10	2.5	3.05×10^{-3}
1	ABED_0094	50S ribosomal protein L13	TL	CYT	-2.8	11	2.6	2.45×10^{-3}
1	ABED_0663	oxidoreductase	ET	UN	-2.5	30	3.0	8.92×10^{-4}
1	ABED_1626	ADP-L-glycero-D-manno-heptose-6-epimerase	CA	UN	-2.2	17	2.6	2.58×10^{-3}
1	ABED_0502	methyl-accepting chemotaxis protein	MC	CM	-2.1	23	3.3	4.57×10^{-4}
1	ABED_1949	DNA-binding ferritin-like protein (Dps/NapA)	RS	CYT	-2.0	155	20.8	1.62×10^{-21}
1	ABED_0457	outer membrane fibronectin-binding protein	AB	OM	-1.9	185	11.4	3.72×10^{-12}
1	ABED_1897	thioredoxin	RS	CYT	-1.8	15	2.6	2.31×10^{-3}
1	ABED_1864	acyl carrier protein, putative	FA	CYT	-1.8	12	3.6	2.41×10^{-4}
1	ABED_1706	50S ribosomal protein L7/L12	TL	CYT	-1.7	71	5.9	1.19×10^{-6}
1	ABED_1980	sodium:sulfate symporter family protein	TB	CM	-1.6	4	2.1	7.28×10^{-3}
1	ABED_1472	peptidyl-prolyl cis-trans isomerase	PF	CYT	1.6	18	2.9	1.21×10^{-3}
1	ABED_1871	ubiquinol cytochrome c oxidoreductase, cytochrome c1 subunit	ET	PP	1.7	48	5.4	3.98×10^{-6}
1	ABED_1045	TonB-dependent receptor	TB	CM	1.9	8	2.1	8.46×10^{-3}
1	ABED_0476	acetate permease	TB	CM	2.0	19	3.5	3.24×10^{-4}
1	ABED_0474	sodium:solute symporter family protein	TB	CM	2.2	8	2.6	2.67×10^{-3}
1	ABED_0704	50S ribosomal protein L22	TL	CYT	2.2	3	1.6	2.51×10^{-2}
1	ABED_1623	50S ribosomal protein L28	TB	UN	2.8	3	1.7	1.99×10^{-2}
1	ABED_0581	TonB-dependent receptor protein	TB	OM	3.0	20	5.1	7.54×10^{-6}
1	ABED_0582	TonB-dependent receptor protein	TB	OM	3.4	46	9.4	4.37×10^{-10}
1	ABED_0477	Predicted membrane protein	HY	CM	3.9	8	6.3	4.84×10^{-7}
1	ABED_1576	putative cytochrome	ET	CM	4.4	8	4.0	9.87×10^{-5}
2	ABED_1706	50S ribosomal protein L7/L12	TL	CYT	-2.5	37	6.5	2.84×10^{-7}
2	ABED_1897	thioredoxin	RS	CYT	-1.9	64	3.1	7.39×10^{-4}
2	ABED_1235	DNA-binding protein HU	NU	UN	-1.9	57	3.9	1.39×10^{-4}
2	ABED_0225	30S ribosomal protein S18	TL	CYT	-1.8	22	3.0	9.34×10^{-4}
2	ABED_1364	serine hydroxymethyltransferase	TF	CYT	-1.8	30	4.0	1.06×10^{-4}
2	ABED_1194	argininosuccinate synthase	IM	CYT	-1.8	22	3.2	5.98×10^{-4}
2	ABED_0457	outer membrane fibronectin-binding protein	CA	OM	-1.6	81	4.1	7.13×10^{-5}

2	ABED_1736	putative citrate lyase	IM	CYT	-1.6	54	4.2	6.29 x 10^{-5}
2	ABED_0272	alkyl hydroperoxide reductase/thiol specific antioxidant	RS	CYT	-1.4	69	3.8	1.48 x 10^{-4}
2	ABED_1343	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	IM	CYT	-1.3	176	4.1	8.41 x 10^{-5}
2	ABED_1338	Ni/Fe-hydrogenase, large subunit	ET	CM	1.4	44	3.3	4.57 x 10^{-4}
2	ABED_0282	fumarate reductase iron-sulfur subunit	ET/IM	CM	1.6	47	5.4	4.00 x 10^{-6}
2	ABED_1484	ATP synthase F0 sector, subunit B	ET	UN	1.6	18	2.8	1.65 x 10^{-3}
2	ABED_1871	ubiquinol cytochrome c oxidoreductase, cytochrome c1 subunit	ET	PP	1.6	37	4.2	7.02 x 10^{-5}
2	ABED_1873	ubiquinol cytochrome c oxidoreductase, 2Fe-2S subunit	ET	CM	1.8	14	2.7	1.83 x 10^{-3}
2	ABED_0476	acetate permease	TB	CM	1.9	20	3.6	2.65 x 10^{-4}
2	ABED_0581	TonB-dependent receptor protein	TB	OM	2.6	22	5.2	5.88 x 10^{-6}
2	ABED_0582	TonB-dependent receptor protein	TB	OM	2.9	37	7.1	8.15 x 10^{-8}
2	ABED_1576	putative cytochrome	ET	CM	3.8	11	3.7	1.90 x 10^{-4}
2	ABED_1575	cytochrome c class I	ET	CM	5.3	4	2.3	4.78 x 10^{-3}

Table 5.4 shows the proteins up-regulated in cells harvested from the anaerobic planktonic phase to cells harvested from aerobic planktonic cultures. Up-regulated proteins are shown in green. Locus tag shows which gene this particular peptide is coded. Functional category denotes which functional category the protein belongs to these are as follows (a) Functional Category: AB – Amino acid biosynthesis; CA – Cell adhesion; CM – Carbohydrate metabolism; ET – Energy metabolism and electron transport; FA – Fatty acid metabolism; HY – Hypothetical; NU – nucleic acid metabolism; MC – Motility and Chemotaxis**;** PH – Protein folding; IM –Intermediary metabolism; RS – Response to stress; TB – Transport proteins; TF – Transferase; TB – Transport proteins; TL – Translation; (b) Localisation: CYT – cytoplasmic; CM – cytoplasmic membrane; OM – outer membrane; EC – extracellular; PP – periplasmic; and UN – unknown, as indicated by PSORT predictions from the genome website (http://www.psort.org/psortb/). (c) Fold; Fold-change and statistical significance are indicated by the score (-log10 of the *p*-value). The p-values (or scores) are included to give an indication of statistical significance.

iTRAQ	Locus Tag	Protein Description	Functional category[a]	Loc.[b]	Fold change Anaerobic planktonic vs aerobic planktonic[c]	No. Peptides	Score[d]	*p*-value
3	ABED_0094	50S ribosomal protein L13	TL	CYT	-3.2	7	4.9	1.29×10^{-5}
3	ABED_1235	DNA-binding protein HU	RS	UN	-2.5	96	4.5	3.35×10^{-5}
3	ABED_1832	50S ribosomal protein L19	TL	CYT	-1.9	18	3.7	2.24×10^{-4}
3	ABED_0476	acetate permease	TB	CM	1.6	37	2.1	7.45×10^{-3}
4	ABED_0165	major facilitator transporter	TB	CM	-4.5	33	5.8	1.76×10^{-6}
4	ABED_0703	30S ribosomal protein S19	TL	CYT	-3.6	4	1.8	1.58×10^{-2}
4	ABED_1884	cytochrome c oxidase, cbb3-type, subunit III	ET	CM	-3.1	31	5.0	1.07×10^{-5}
4	ABED_0710	50S ribosomal protein L24	TL	CYT	-2.2	28	4.1	8.36×10^{-5}
4	ABED_1886	cytochrome c oxidase, cbb3-type, subunit II	ET	PP	-2.1	75	7.8	1.44×10^{-8}
4	ABED_1974	conserved hypothetical protein	HY	EC	-2.1	219	13.0	1.05×10^{-13}
4	ABED_1883	conserved hypothetical protein	HY	CM	-1.8	19	3.0	1.10×10^{-3}
4	ABED_0241	pyruvate kinase	IM	CYT	-1.7	18	2.3	5.13×10^{-3}
4	ABED_1484	ATP synthase F0 sector, subunit B	ET	UN	-1.6	56	2.7	1.92×10^{-3}
4	ABED_0282	fumarate reductase iron-sulfur subunit	ET/IM	CM	-1.5	51	2.5	3.27×10^{-3}
4	ABED_0457	outer membrane fibronectin-binding protein	ET/IM	OM	-1.4	198	6.0	9.22×10^{-7}
4	ABED_0283	fumarate reductase flavoprotein subunit	ET/IM	CM	-1.4	119	4.4	3.81×10^{-5}
4	ABED_1482	F0F1 ATP synthase subunit alpha	ET	CYT	-1.2	392	6.9	1.41×10^{-7}
4	ABED_1706	50S ribosomal protein L7/L12	TL	CYT	-1.1	229	1.9	1.41×10^{-2}
4	ABED_1797	30S ribosomal protein S7	TL	CYT	1.3	92	2.1	8.29×10^{-3}
4	ABED_0715	50S ribosomal protein L18	TL	CYT	1.4	32	2.0	1.07×10^{-2}
4	ABED_1864	acyl carrier protein, putative	FA	CYT	1.5	65	2.2	5.98×10^{-3}
4	ABED_0293	citrate synthase	IM	CYT	1.6	94	4.5	3.29×10^{-5}
4	ABED_1343	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	IM	CYT	1.6	126	3.6	2.25×10^{-4}
4	ABED_1737	fumarate hydratase, class I	IM	CYT	1.8	27	1.9	1.38×10^{-2}
4	ABED_0260	methylcitrate synthase	IM	CYT	1.8	28	2.4	4.05×10^{-3}
4	ABED_1944	acetolactate synthase	IM	CYT	1.8	35	3.0	8.93×10^{-4}
4	ABED_1986	hypothetical protein	HY	UN	2.0	16	1.8	1.48×10^{-2}
4	ABED_0272	alkyl hydroperoxide reductase/thiol specific antioxidant	RS	CYT	2.1	51	5.4	3.95×10^{-6}
4	ABED_1713	elongation factor Tu	TL	CYT	2.1	125	5.4	3.80×10^{-6}

4	ABED_2065	flagellin	MC	UN	2.3	209	7.2	5.87×10^{-8}
4	ABED_1964	O-acetylhomoserine sulfhydrylase	AB	CYT	2.4	10	1.6	2.40×10^{-2}
4	ABED_1364	serine hydroxymethyltransferase	T	CYT	2.5	17	2.3	4.90×10^{-3}
4	ABED_0259	2-methylisocitrate lyase	IM	CYT	3.4	14	2.4	3.55×10^{-3}

Table 5.5 shows the proteins up-regulated in cells harvested from the anodic phase to cells harvested from aerobic planktonic cultures. Locus tag shows which gene this particular peptide is coded, functional category shows which functional category the protein belongs to these are as follows (a) Functional Category: AB – Amino acid biosynthesis; CA – Cell adhesion; CM – Carbohydrate metabolism; ET – Energy metabolism and electron transport; FA – Fatty acid metabolism; HY – Hypothetical; NU – nucleic acid metabolism; MC – Motility and Chemotaxis**;** PH – Protein folding; IM –Intermediary metabolism; RS – Response to stress; TB – Transport proteins; TF – Transferase; TB – Transport proteins; TL – Translation; (b) Localisation: CYT – cytoplasmic; CM – cytoplasmic membrane; OM – outer membrane; EC – extracellular; PP – periplasmic; and UN – unknown, as indicated by PSORT predictions from the genome website (http://www.psort.org/psortb/). (c) Fold; Fold-change and statistical significance are indicated by the score (-log10 of the *p*-value). The p-values (or scores) are included to give an indication of statistical significance

5.2.4 Imaging of *A. butzleri* ED-1 by scanning electron microscopy

1 cm^2 sections of anode were cut and imaged by scanning electron microscopy (SEM).10 mL samples of aerobic planktonic culture were also taken and dropped onto 25 mm by 25 mm poly-l-lysine coated coverslips in order to be imaged by SEM. The samples were prepared according to the protocol described in section 6.2. This was done in order to see if there were any morphological differences between

anode associated *A. butzleri* ED-1 and aerobic, planktonic *A. butzleri* ED-1. Figure 5.3 shows the images of anode associated and aerobic planktonic *A. butzleri* ED-1.

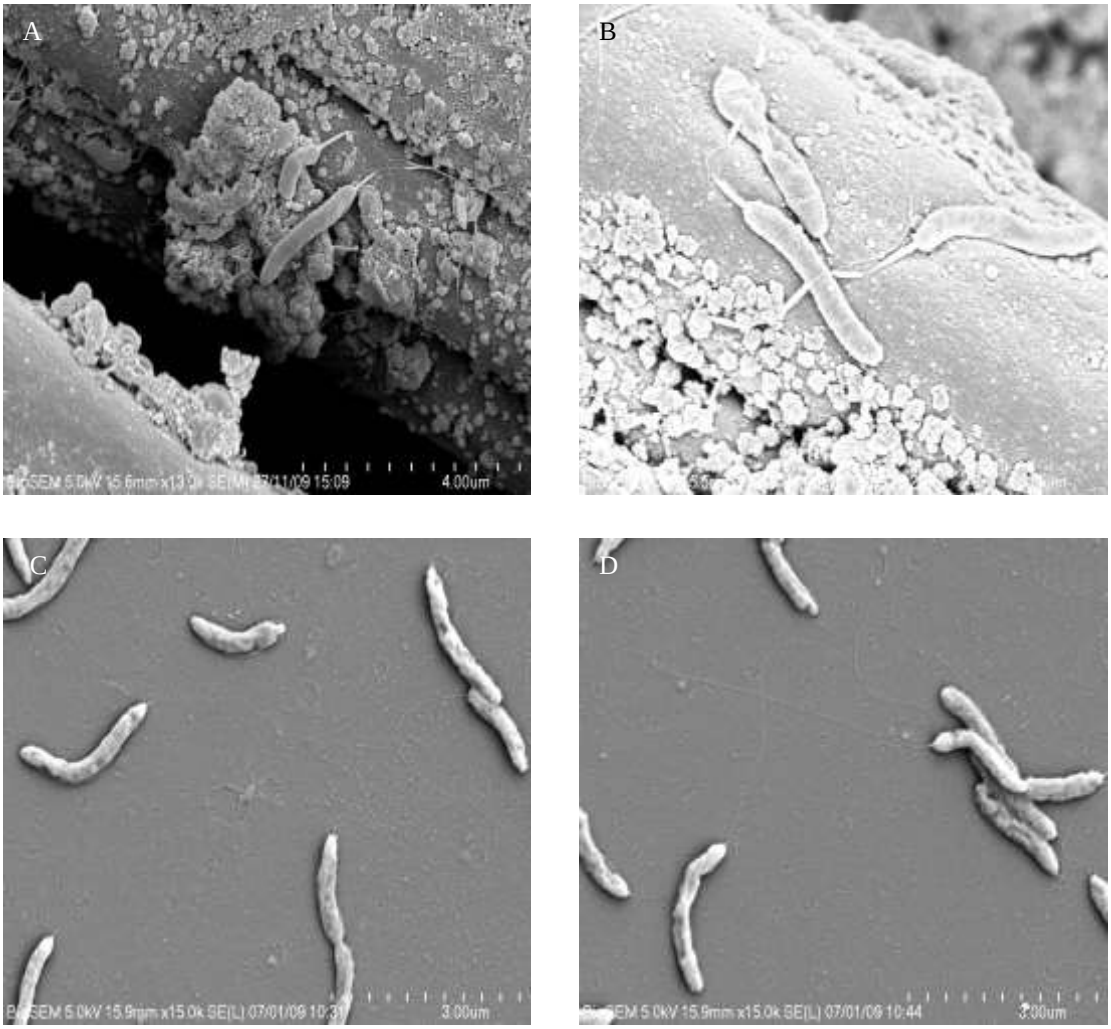


Figure 5.3 shows SEM images of *A. butzleri* ED-1 grown on the anode after 336 h (A-B) or images of cells harvested from the anodic planktonic phase after 72 h (C-D). Cells prepared for imaging by a modified version of the protocol used by Gorby *et al*. 2006. Flagella of cells associated from the anode display an altered morphology of shorter flagella with thicker and thinner sections. Cells from aerobic, planktonic cultures have longer, thinner flagella with a "whip like" morphology. The images of anode associated *A. butzleri* ED-1 displayed were taken from a selection of 50 images, the images of aerobic planktonic *A. butzleri* ED-1 displayed were taken from a selection of 20 images.

The length and thickness of the flagella was measured for the aerobic planktonic images and for the anode associated images. The measurements are shown in Table 5. 6

Appendage	Mean length (μm)	Mean thickness (μm)
Aerobic planktonic flagella	4.3 ± 0.78	0.004 ± 0.004
Anode associated flagella	1.81 ± 0.702	n/a (2 sections of different thickness, thus measured separately)
Thick section of anode associated flagella	0.46 ± 0.148	0.085 ± 0.02
Thin section of anode associated flagella	0.86 ± 0.57	0.03 ± 0.09

Table 5.6 shows the mean lengths and thickness of the flagella of both anode associated cells and aerobic planktonic cells. The mean and standard deviation was calculated from measurements taken from across a sample of 50 images for anode associated bacteria and 20 images for aerobic planktonic bacteria.

A clear difference in length was observed between flagella from aerobic and anode associated cells with the flagella from aerobic cells being double the length of those observed on the anode. The anode-associated cells were divided into thick sections, which were over the double the thickness of the thin sections but approximately half the length. It is critical to note the difference was not an artefact of sample preparation as both sets of samples were prepared identically using the protocol

given by Gorby *et al*., which was used to image *Shewanella* expressing nanowires (Gorby *et al*., 2006), and therefore is unlikely to damage extracellular appendages (The Gorby study did not report any damaged to nanowires as a result of sample preparation).

5.3 Discussion

5.3.1 Development of a screen for electrogenesis using redox indicators and metal oxides

A.butzleri ED-1 was found to reduce Nile blue and Indigocarmine whereas *E. coli* did not and Methylene blue was reduced by both *E. coli* and *A. butzleri* ED-1 presumably due to its high E◦V, which was to be expected as methylene blue reduction is a test as to whether a cell is alive or not (living cells reduce the methylene blue from blue to colourless).

Despite the differences observed with Nile blue and indigocarmine the dyes were deemed unsuitable for a screen as the areas of colour change were not limited to a single colony and so could not be used to determine whether a single colony had impaired electrogenic ability or not and so could not be used to screen for transposon mutants with impaired electrogenic ability. The same differences in colour change were also observed when the bacterium was cultured in liquid medium. One possible screening method would have been to screen bacteria by culturing them in micro-titre plates using medium with 1 mM of Nile blue or indigocarmine added.

When grown with FeOOH and ferric citrate no visible colour change was observed which would indicate the reduction of Fe^{3+} to $Fe^{2+.}$ This screen was also repeated with ferrozine added, however this did not produce a visible colour change for *A. butzleri* ED-1 and produced a colour change for *E. coli* cultures before incubation due to Fe^{2+} already being present in the medium. The two most likely reasons for the lack of observed colour change were an insufficient incubation time; the only time a colour change was observed for *A. butzleri* ED-1 incubation on FeOOH was after one month or that *A. butzleri* ED-1 was not capable of using Fe^{3+} as a terminal electron acceptor. In any case FeOOH and ferric citrate were deemed unsuitable as a potential screen for electrogenic ability.

It was decided that redox indicators and metal oxides could not be used as an effective screen for electrogenesis and therefore a transposon mutagenesis based method could not be used to investigate the electrogenic mechanisms of *A. butzleri* ED-1. Therefore it was decided to use the proteomics based method for reasons discussed in section 5.1.

5.3.2 Growth of cultures for iTRAQ analysis

The drop to a negative redox potential was much quicker than previously observed presumably (see chapter 3, section 3.6) due to the basal AMM being deoxygenated by a vacuum pump than pre-reduced by incubating in an anaerobic atmosphere

overnight (see chapter 2, section 2. 2.1), which meant there was no dissolved oxygen in the basal medium. The variation in end point redox potentials and variation in decline of redox potentials is not unusual amongst *A. butzleri* ED-1 half MFC cultures, which have previously exhibited such variation when grown in pure culture (purity of culture was regularly checked by light microscopy). One explanation of this irregularity is that minute variations on the anode surface influence how the bacteria interact with each individual anode and therefore affect the redox potential generated.

The growth of *A. butzleri* ED-1 was much higher than previously observed (see chapter 3) with cultures reaching A_{600} of approximately 0.819±0.173. This was due to the intensive feeding regimen as after 96 h of growth cultures were supplied with 20 mL of fresh AMM (pH 6.5) containing 30 mM acetate as the sole carbon source . The growth of cells in the planktonic phase was unusually high considering that there are no apparent terminal electron acceptors in the growth medium. The high number of cells in the planktonic phase has been seen in previous half MFC cultures and it was confirmed not be due to contamination by light microscopy. The high number of planktonic cells grown under strict anaerobic conditions suggests that the growth is not due to imperfect seals meaning that small amounts of dissolved oxygen are present in the planktonic phase (the other reason thought to be behind the reason for high numbers of cells in the planktonic phase of the half MFC) but rather transfer of electrons to the anode by mediator compounds. Previous studies suggested that *A. butzleri* ED-1 did not produce mediators (Fedorovich, personal communication) although this should be repeated now that a more defined minimal medium is in use.

As discussed in chapter 1 *Shewanella oneidensis* MR-1 possible performs electrogenesis by both direct contact and mediators.

Therefore it was concluded that high growth yield observed in the half MFC cultures was due to the intensive redox potential and the much rapider decrease in redox potential observed was due to deoxygenation of the basal medium used. The high amount of cells in the planktonic phase of the half MFC was possibly due to *A. butzleri* ED-1 transferring electrons to the anode by mediator compounds although further study is required to explore this possibility further.

5.3.2 up-regulation of FlaA at the anode

The flagellin protein, FlaA was found to be up-regulated 2.4 fold at the anode when compared to cells grown aerobically and from the planktonic phase of the half MFC. In one respect this was unsurprising since the up regulation of flagellin has been observed in biofilm formation amongst a number of species including *Campylobacter jejuni* (Joshua *et al*., 2006, Kalmokoff *et al*., 2006), *Pseudomonas aeruginosa* (O'Toole and Kolter 1998) and *Listeria monocytogenes* (Lemon *et al*., 2007). The closely related nature of *C. jejuni* and *A. butzleri* mean that it is likely that flagella play a similar role in *Arcobacter* biofilm formation. The up-regulation of FlaA as opposed to FlaB at the anode is logical as FlaA has been shown to be essential for *A. butzleri* motility (Ho *et al*., 2008) and in all the above studies motility was found to be essential to biofilm formation.

Campylobacter jejuni has been found to express higher levels of flagellin and other motility associated proteins under severely oxygen-limited conditions (Gaynor *et al*., 2004). In this particular study the putative flagellin, FlgB, exhibited a 7-fold up-regulation under oxygen-limited conditions (Gaynor *et al*., 2004). *Campylobacter* have also been found to be more motile during the stationary phase of growth, when grown on acetate (Write *et al*., 2009). Therefore it is possible that the up-regulated FlaA observed here is due to the *Arcobacter* being in stationary phase and a response to the high levels of acetate supplied to the system.

A third reason for the up-regulation of FlaA at the anode is that the protein is possibly involved in facilitating the transfer of electrons from the cells to the anode. There is precedent for the involvement of flagella in the electrogenic process. Highly electrogenic variants of *Geobacter sulfurreducens* have been shown to express flagella (Yi *et al*., 2009). In this study *Geobacter sulfurreducens* were cultured in a system poised at – 400 mV in order to select for bacteria with the most efficient extracellular electron transfer pathways as this voltage would not favour the transfer of electrons to the anode. It was found that the bacteria isolated from this system after 5 months of growth produced flagella (which the type strain does not) and produced a greater number of nanowires (Yi *et al*., 2009). The strain isolated from the system (*Geobacter sulfurreducens* KN400) produced higher current and power densities than the type strain (*Geobacter sulfurreducens* DL1) generated current and power density of 7.6 A/m^2 and 3.9 W/m^2 respectively. This was substantially higher than the current and power density generated by the type strain which was 1.4 A/m^2 and 0.5 W/m^2 although *Geobacter sulfurreducens* KN400 was found to express less

outer membrane c type cytochromes than DL1 (Yi *et al*., 2009). Therefore it is possible that the expression of flagella resulted in a more electrogenic variant of *Geobacter sulfurreducens,* although there are other reasons such as the less internally resistant nanowires of KN400 (Yi *et al*., 2009) and further study of the strain suggests that the increased electrogenic ability is not due to novel gene acquisition (*Geobacter sulfurrdeucens* KN400 was found to several genes not found in the type strain including a metal-transporting ATPase, a metal dependent phosphohydrolase and a restriction modification system) but rather changes in common metabolic pathways resulting in an increase in the rate of electron transfer due to a shift towards oxidation pathways and a change in ATP production (Butler *et al*., 2012). Therefore it is unlikely that the presence of flagella results in increased electrogenesis, although the role of flagella in KN400 electrogenesis is yet to be fully explored.

The altered morphology displayed by anode associated flagella also suggests that the flagella may be the result of anode interaction, although whether this is due to biofilm formation or electron transfer to the anode remains unknown. One possible way to investigate this would be to perform SEM *A. butzleri* ED-1 biofilms grown aerobically on the anode, aerobically and anaerobically on a glass coverslip and compare the morphology of *A. butzleri* ED-1 flagella under these conditions to see whether or not the flagella display altered morphology only when associated with a half MFC anode.

Based on the precedent established by other studies the most likely reason for the up-regulation of FlaA in *A. butzleri* ED-1 associated with the anode is that it plays a role in attachment to and biofilm formation on the anode. It is possible that the up-regulation of FlaA is due to it playing a role in extracellular electron transfer, although this cannot be confirmed without further study and previous studies in *Geobacter* suggest this is unlikely.

The role of FlaA should be investigated both by comparing the proteome of the anodic biofilm to the proteomes of *A. butzleri* ED-1 grown aerobically on the anode and anaerobically using a soluble terminal electron acceptor to show whether it is up-regulated more in all types of biofilm or just anaerobic- anodic biofilms (which would suggest a role in extracellular electron transfer). Deletion mutants of FlaA should also be constructed in order to explore how lack of FlaA effects generation of electricity and biofilm formation.

5.3.3 No electron transport proteins were up regulated at the anode

No electron transport proteins were up regulated in cells harvested from the anodic biofilm and in the case of cytochrome c oxidase cbb3 (ABED_1884/ABED_1886) and fumarate reductase (ABED_0282) was actually down regulated at the anode. The down regulation of cytochrome c oxidase cbb3 is unsurprising as the protein is typically involved in aerobic pathways of electron transfer and so is unlikely to be involved in anaerobic respiration. The down regulation of fumarate reductase is

somewhat unexpected as fumarate reductase is used in anaerobic respiration where fumarate is serves as a terminal electron acceptor; as such an up regulation may be expected under anaerobic conditions such as the half MFC. However it must be noted that fumarate is a soluble terminal electron acceptor, whereas the anode is an insoluble electron acceptor so the pathways involved are likely to be distinct.

A. butzleri encodes for a full set of genes for aerobic/ microaerobic respiration including NADH: quinone oxidoreductast, ubiquinol cytochromes c oxidase, ferredoxin, cytochromes *bd* oxidase, cytochromes c oxidase (*cbb*3-type) and F1/F0 ATPase (Miller *et al*., 2007, Ler 2009). The bacteria also encodes for a number of genes important in anaerobic respiration including fumarate reductase, and nitrate reductases, found in an operon analogous to the *nap* operon found in *Campylobacter* species (Miller *et al*., 2007, Ler 2009). The genome has also been found to contain the c type cytochromes TorA and TorC allowing it to utilise TMAO as an electron acceptor (Miller *et al*., 2007, Ler 2009). The bacterium also encodes for the reductase DmsA allowing it to use DMSO as a terminal electron acceptor and a b type nitrite/ nitric oxide reductase has also been identified (Miller *et al*., 2007, Ler 2009). . Since none of these proteins were up-regulated at the anode it suggests none of the electron transport or putative electron transport proteins are involved in extracellular electron transfer.

It is surprising that no electron transport proteins are up regulated at the anode as previous studies have shown that electron transporter proteins are up regulated in the anodic biofilm (Nevin *et al*., 2009). In this study transcriptomic analysis of an

anodic *Geobacter sulfurreducens* compared to a fumarate grown *Geobacter sulfurreducens* biofilm showed that the outer membrane c type cytochromes OmcB and OmcZ were up-regulated along with the PilA protein in bacterium harvested from an anodic biofilm. Transcriptomic analysis of *Shewanella oneidensis* MR-1 showed similar results i.e. the up-regulation of outer membrane c type cytochromes in bacteria harvested from the anode (Rosenbaum *et al*., 2012). Therefore if *A. butzleri* ED-1 were performing electrogenesis in a fashion similar to *Geobacter sulfurreducens* or *Shewanella oneidensis* it would be expected to see electron transport proteins up-regulated in *A. butzleri* ED-1 harvested from the anode. It must be noted that electron transporter proteins may be involved in transporting electrons across the anode and are simply not up-regulated in comparison to the proteomes of cells harvested from anaerobic or aerobic planktonic cultures.

The lack of up-regulation suggests that *A. butzleri* ED-1 may perform electrogenesis in a significantly different fashion from either *Geobacter sulfurreducens* or *Shewanella oneidensis* although further study is required before this is confirmed and new electrogenic mechanisms are elucidated for the bacterium. Such further studies would involve the comparison of proteomes from *A. butzleri* ED-1 harvested from aerobic biofilm and from bacteria grown anaerobically with a soluble terminal electron acceptor. This would possibly more use in highlighting whether any electron transporter proteins are up-regulated at the anode than the current comparison which did not compare the proteomics of non-anodic *A. butzleri* ED-1 biofilms. More stringent studies into whether or not the bacteria produces mediators must also be performed in order to determine how *A. butzleri* ED-1 performs electrogenesis.

5.3.4 No adhesins were up regulated at the anode

There was no evidence of any other adhesins being up regulated at the anode with the fibronectin binding protein (ABED_0457) being down regulated at the anode. This was to be expected as fibronectin is a very different surface from a carbon anode and it is unlikely that the protein would adhere well to the carbon surface. *A.butzleri* contains many other adhesins (Miller *et al*., 2007, Toh *et al*., 2011), none of which were found to be up-regulated at the anode. Much like in *Campylobacter jejuni* biofilm formation in *A. butzleri* ED-1 is likely to be mediated by flagella, LPS, capsular polysaccharides and glycoproteins (Joshua *et al*., 2006). The lack of up-regulation of other adhesins further suggests that the up-regulation of FlaA is due to its role in biofilm formation rather than a role in extracellular electron transfer; although further study is required as described above.

Geobacter sulfurreducens and *Shewanella oneidensis* MR-1 both showed up-regulation of the adhesin PilA when harvested from the anode of an MFC (Nevin *et al*., 2009, Rosenbaum *et al*., 2012). As previously discussed in chapter 1 PilA is important in the formation of nanowires and the electrically conductive anodic biofilm, but not essential for the adhesion of the bacteria to the anode (Reguera *et al*., 2005, Reguera *et al*., 2006, Gorby *et al*., 2006). *A. butzleri* ED-1 does not contain homologs for PilA but does contain other adhesins (Toh *et al*., 2011) none of which are-upregulated in the anode. PilA are thought to be an essential part of the electrogenic process in *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1

therefore the lack of up-regulation of any proteins that could fulfil a similar role in *A. butzleri* ED-1 suggests that *A. butzleri* ED-1 may perform electrogenesis in a very different fashion to *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1. It must be noted that the proteomes of anodic, non-anodic and aerobically grown anodic biofilms must be compared in order to gain a better idea of what proteins are potentially important to electrogenesis.

Therefore the lack of up-regulation of adhesins suggests that flagella and molecules such as LPS are important in *A. butzleri* ED-1 biofilm formation and that the bacterium performs electrogenesis by a different method than that described for *Geobacter sulfurreducens* and *Shewanella oneidensis,* although further study is required to confirm this conclusion and fully explore on the electrogenic processes of *A. butzleri* ED-1.

5.3.5 Up-regulation of enzymes of the methyl-citrate cycle at the anode

The methylcitrate cycle is a biochemical pathway that replenishes pyruvate and oxaloacetate (Ler 2009). There are three enzymes specific to the methylcitrate cycle; methylcitrate synthase, methylcitrate dehydratase and methylisocitrate lyase. When *A. butzleri* ED-1 was grown at the anode it was found that methylcitrate synthase (ABED_0260), bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase (ABED_1343), and 2-methylisocitrate lyase (ABED_0259) were up regulated.

The up regulation of methylcitrate cycle proteins was most likely due to the high

amounts of acetate supplied to the cell due to the intense feeding regimen previously discussed. The increased uptake of acetate is shown by the up regulation of acetate permease (ABED_0476) 1.6 fold at the anode. The increased concentration/ uptake of acetate resulted in an up regulation of the above enzymes of the methyl citrate cycle. The interactions of the methyl citrate cycle and acetate metabolism are shown in Figure 5.4.

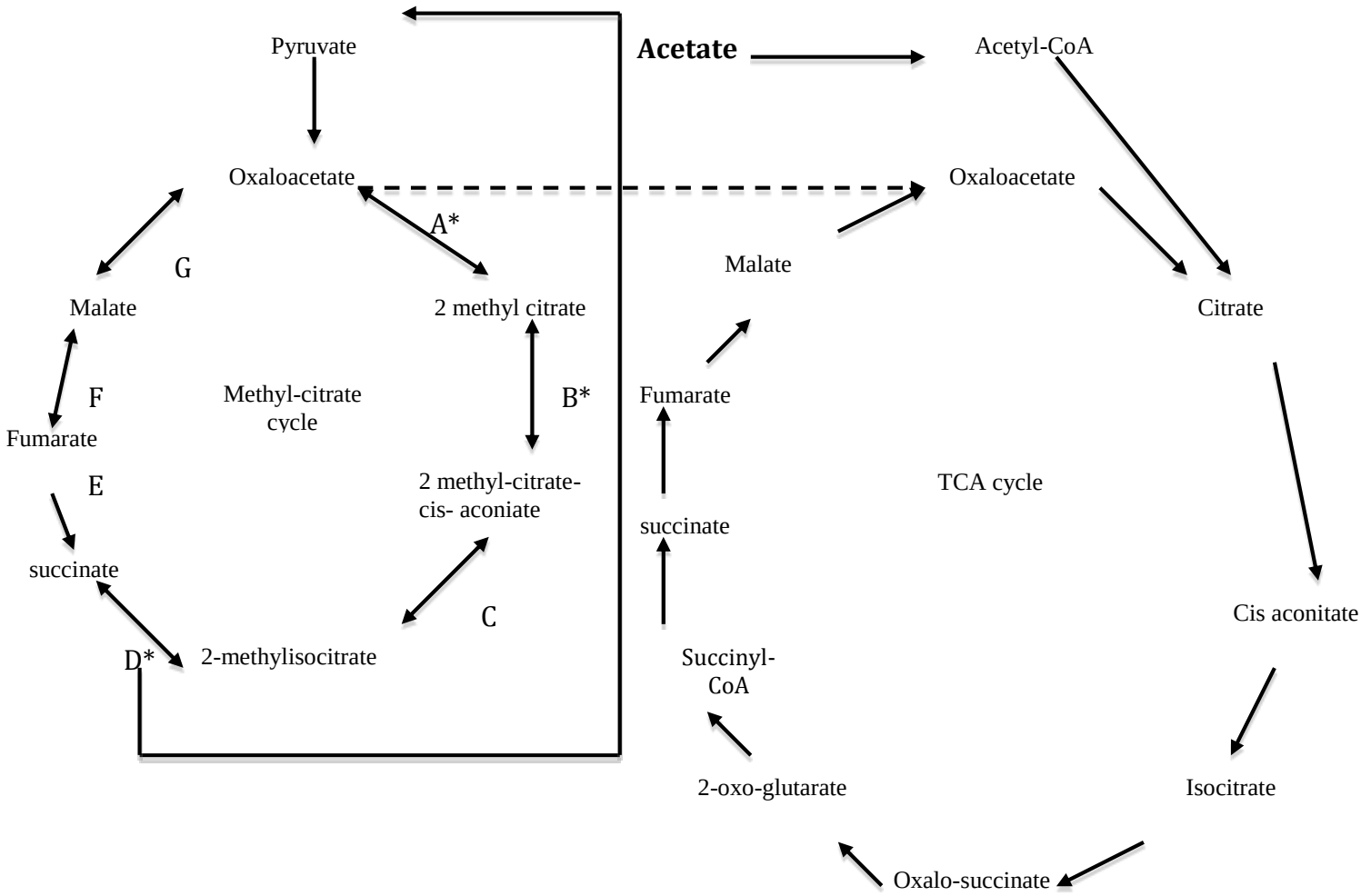


Figure 5.4 adapted from Ler 2009. It shows the Methyl-citrate cycle (left) and the TCA cycle (right) with regards to acetate metabolism. The enzymes of the methyl-citrate cycle are 2 Methylcitrate synthase (A), Aconitate hydratase (B), 2-Methylisocitrate dehydratase (C), Methylisocitrate lyase (D), Fumarate reductase (E), Fumarate Hydratase (F) and Malate dehydrogenase (G). The enzymes marked with * are up-regulated. The Methylcitrate cycle generates oxaloacetate, which feeds into the TCA cycle. Acetate enters the TCA cycle as acetyl-CoA which reacts with oxaloacetate to form citrate. Therefore an increased level of acetate requires an increase in oxaloacetate and the enzymes up-regulated result in more oxaloacetate being generated. Up-regulated Methylisocitrate lyase also generates pyruvate, which is converted into more oxaloacetate, thus further satisfying the requirement.

Figure 5.4 shows how the enzymes of the methylcitrate cycle are up regulated when the concentration of acetate supplied to the bacteria is increased. The most likely explanation for this is that the methyl-citrate cycle regenerates oxaloacetate that feeds into the TCA cycle. This oxaloacetate then reacts with acetate to form citrate. Therefore higher levels of acetate require a higher level of oxaloacetate so aconitate hydratase, methylisocitrate dehydratase and methylisocitrate lyase are up regulated to regenerate oxaloacetate at a more rapid rate. The iTRAQ analysis therefore validates the pathway of acetate metabolism suggested by the metabolic reconstruction (Ler 2009).

It must be noted that the bacteria in the planktonic phase of the half MFC did not show the same up regulation of methylcitrate cycle enzymes. One suggestion for this is that the consumption of acetate is linked to biofilm formation. Studies have shown that in *C. jejuni* the consumption of acetate acts as a metabolic switch to express genes for biofilm formation and motility (Write *et al*., 2009) and *A. butzleri* ED-1 anode biofilms do show an increased expression of FlaA (see section 5.3.2 for more details) which is associated both with biofilm formation (Joshua *et al*., 2006).

However the high growth of *A. butzleri* ED-1 in the planktonic phase suggests that the bacterium is consuming acetate as that was the only available carbon source for growth (see section 5.2). The reason for the lack of up regulation is unknown.

One possible reason is that the bacteria are growing without the lack of a solid electron acceptor or that it is interacting with anode via mediator compounds. It

must be noted that there were no known soluble electron acceptors added to the medium and as previously discussed there is no evidence for the use of mediator compounds by *A.butzleri* ED-1 One possible explanation for this is that the bacteria are using soluble $Fe^{3+.}$ The Fe^{2+} supplied in the form of Fe_2SO_4, has been observed to partially oxidise over time (as indicated by a small amount of brick red precipitate) and therefore Fe^{3+} would be present in the medium, which *A. butzleri* ED-1 could theoretically use as an electron acceptor. The simplest way to test this would be to grow *A. butzleri* ED-1 with and without a source of Fe^{2+} and compare the proteomes.

5.4 Conclusions

From the iTRAQ proteomic analysis performed it can be concluded that the up-regulation of FlaA is most likely due to its role in biofilm formation,. It is possible that FlaA is involved in electrogenesis, although this requires further study and there is little precedent in the literature for it to play such a role.

The lack of up-regulation of electron transporter proteins and adhesins in *A. butzleri* ED-1 harvested from the anode suggests that *A. butzleri* ED-1 has distinct electrogenic mechanisms to *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1; both of which show up-regulation of electron transporter proteins (c type cytochromes) and PilA adhesins when harvested from the anode. However further studies must be performed comparing the proteomes of *A. butzleri* ED-1 biofilms to the proteomes of aerobic and anaerobic, non-anodic biofilms to explore this further.

Finally it can be concluded from the up-regulation of enzymes of the methyl-citrate cycle that acetate metabolism occurs by the pathway predicted in the metabolic reconstruction (Ler 2009).

Chapter 6

The attempted construction of *fla*A and *fla*B deletion mutants *in A. butzleri* ED-1

6.1 Introduction

The flagellin protein, FlaA, was found to be up-regulated at the anode 2.4 fold when compared to cells harvested from aerobic planktonic cultures. It was therefore concluded that FlaA possessed a role in biofilm formation and/or a role in extracellular electron transfer to the anode by *A. butzleri* ED-1 (see chapter 5). It was decided to investigate the role of FlaA in *A. butzleri* ED-1 biofilm formation and extracellular electron transfer by creating deletion mutants of the *fla*A and *fla*B genes. The two genes would be deleted together as *fla*A and *fla*B lie close to each other in the genome (Toh *et al*., 2011) and therefore it was deemed simpler to delete both genes together. The location of the *fla*A and *fla*B genes of *A. butzleri* ED-1 corresponds to the analogous genes in *C. jejuni* i.e. the order in which they appear in the annotation of the genome in both bacteria is *fla*B is upstream of *fla*A and separated by a small region of about 100 bp.

Deletion mutants have been used to study many different properties such as motility, biofilm formation and virulence in a wide variety of bacteria. Of particular interest to this study is the use of deletion mutants in *Campylobacter spp*. to study flagella and a variety of other genes, typically virulence factors due to the importance of the bacteria as a human pathogen. Genetic work in *Campylobacter spp*. is important resource for the genetic manipulation of *Arcobacter spp.* as there is a distinct lack of genetic tools for working with *Arcobacter spp.*

To date there has only been one study using deletion mutants in *A. butzleri.* This was to investigate the role of *fla*A and *fla*B in *A. butzleri* motility (Ho *et al*., 2008). This study and the more numerous studies performed in *Campylobacter spp*. are detailed below.

6.1. 1. Previous studies using deletion mutants in *Arcobacter spp*. and *Campylobacter spp.*

Due to their importance as human pathogen *Campylobacter spp. (*particularly *C. jejuni)* have been studied much more intensively then *Arcobacter spp.* and as such deletion mutants have been used to study the role of a number of virulence factors and other proteins in *Campylobacter spp.* including flagella.

The importance of flagella to motility in *Campylobacter spp*. was first shown in 1991 (Gurrey *et al*., 1991). This study showed that FlaA is essential for the formation of a fully functional flagellum, with mutants expressing FlaA alone forming flagella near indistinguishable from wild type flagella, with only a slight decrease in motility (Gurrey *et al*., 1991). By contrast mutants only expressing FlaB were immotile and displayed highly truncated flagella (Gurrey *et al*., 1991). Flagella deletion mutants have also shown the importance of flagella in the colonisation of chicks by *C. jejuni* (Wassaner *et al*., 1993). In this study strains lacking *fla*A genes colonised chicken cells 100-1000 fold less efficiently than wild type bacteria (Wassaner *et al*., 1993) Flagella have also been shown to be important in the colonisation of other animals

where a non-flagellated *C. coli* mutant was unable to colonise rabbits (Pavlovskis *et al*., 1991).

Campylobacter spp. flagella have also been shown to be important in the secretion of effector molecules, such as the *Campylobacter* invasion antigens or Cia molecules (Konkel *et al*., 2004). Deletion mutants of the various components of the flagella, the flagellin proteins *fla*A and *fla*B, the basal components *flgB* and *flg*C and the hook *flg*E2 have all been constructed and their effect on the secretion of Cia molecules investigated (Konkel *et al*., 2004). Mutants lacking both filament components (i.e *fla*A and *fla*B) were found to be incapable of secreting Cia molecules, although a *fla*A^- but *fla*B^+ was found to be capable of secreting Cia molecules (Konkel *et al*., 2004). This suggests that FlaB is the important component of the flagella for the secretion of effector molecules.

Therefore the use of deletion mutants has shown the flagella of *Campylobacter spp*. to be important in both bacterial motility and as a virulence factor. In particular, *fla*A has been shown to be essential for motility and adhesion, whereas *fla*B has been shown to be essential for the secretion of virulence factors such as Cia effector proteins.

Deletion mutants have also been used to show the roles of *Campylobacter spp*. virulence factors. Cytolethal distending toxin is one of the most important *Campylobacter* virulence factors encoded for by three highly conserved genes: *cdt*A,B and C (AbuOun *et al*., 2005). The toxin causes direct DNA damage to the

host cell, which causes the cells to arrest in G1 or G2 and die (Lee *et al*., 2003). Deletion mutant studies have shown the subunits CdtA and CdtC to be important in binding to HeLa cells and have a crucial role in holotoxin formation (Lee *et al*., 2003). Deletion mutants have also been used to show the role of virulence factors such as JlpA in adherence to host cells during *Campylobacter* infection. JlpA deletion mutants showed decreased adherence HEp-2 epithelial cells (Jin *et al*., 2001). The virulence factor Cj1496c was shown to be important in invasion as mutants lacking this glycoprotein were found to be defective for invasion in INT-407 cells (Kakuda and DiRita 2006).

There have been substantially fewer studies involving deletion mutants in *Arcobacter spp*. however the studies performed are detailed below. Ho *et al*., showed that FlaA was essential for motility in *Arcobacter butzleri* (Ho *et al*., 2008) and deletion of *fla*A drastically decreased motility and increased the transcription of *fla*B (Ho *et al*., 2008). No other deletion mutant studies in *Arcobacter* spp. have been reported; therefore it is unknown whether the flagella of *Arcobacter spp*. play a similar role in virulence or the secretion of effector molecules as they do in *Campylobacter spp*.

6.1.2 Strategies for the creation of *A. butzleri* ED-1 deletion mutants

The flagella deletion mutants created by Ho *et al*. could not be used to investigate the role of flagella in *A. butzleri* ED-1 electrogenesis as *A. butzleri* LMG (the strain used by Ho *et al*.) was found to be less electrogenic than *A. butzleri* ED-1 (data not

shown). Therefore deletion mutants of *A. butzleri* ED-1 needed to be constructed. The first step in this process was the construction of a suitable suicide vector.
In order to construct a suitable suicide vector for the creation of deletion mutants' three components were required; a suitable selective marker, a plasmid that did not replicate in *A. butzleri* ED-1 and could be successfully introduced into *A. butzleri* ED-1 by triparental mating the only method that successfully introduced DNA into *Arcobacter spp*. within this project (see chapter 4).

The antibiotic sensitivities of *A. butzleri* ED-1 and *Arcobacter* L were tested using a series of antibiotic discs placed atop a confluent lawn of cells in order to observe zones of clearing that would indicate antibiotic sensitivity (Fedorovitch *et al*., 2009). *A. butzleri* ED-1 was found to sensitive to kanamycin, gentamycin, streptomycin, erythromycin and tetracycline (Fedorovitch *et al*., 2009). *A. butzleri* ED-1 was found to be resistant to chloramphenicol which meant that the suicide vector constructed by Ho *et al*., could not be used as it used chloramphenicol resistance as its selective marker.

Two strategies for the creation of a suicide vector and subsequent creation of *A. butzleri* ED-1 flagella deletion mutants were developed.

6.1.2.3 Creation of a suicide vector using Kanamycin resistance and pBBRMCS-5 (strategy 1)

The first strategy was to use the plasmid pBBR1MCS-5. (Kovach *et al*., 1995). This plasmid was chosen for several reasons. Firstly it has an MCS with restriction sites for easily available enzymes and therefore was suitable for cloning purposes. Secondly it had an *rk*2 based *ori*T, which was compatible with the available helper plasmid pRK600 and therefore could be mobilised by triparental mating. The plasmid came with ColE1 and p15a based replicons (Kovach *et al*., 1995) which are not active in *Campylobacter spp.* and *Arcobacter spp*. The plasmid also came with a gentamycin resistance selective marker which would be useful in screening for mutants after triparental mating i.e. *A. butzleri* ED-1 resistant to kanamycin but sensitive to gentamycin would be assumed to have taken up the insert and lost the plasmid. With these qualities and the fact the plasmid was readily available it was decided to use pBBR1MCS-5 as the basis for the construction of a suicide vector for the creation of *A. butzleri* ED-1 flagella deletion mutants; the vector would be named pMKsui and constructed by the strategy illustrated in Figure 6.1.

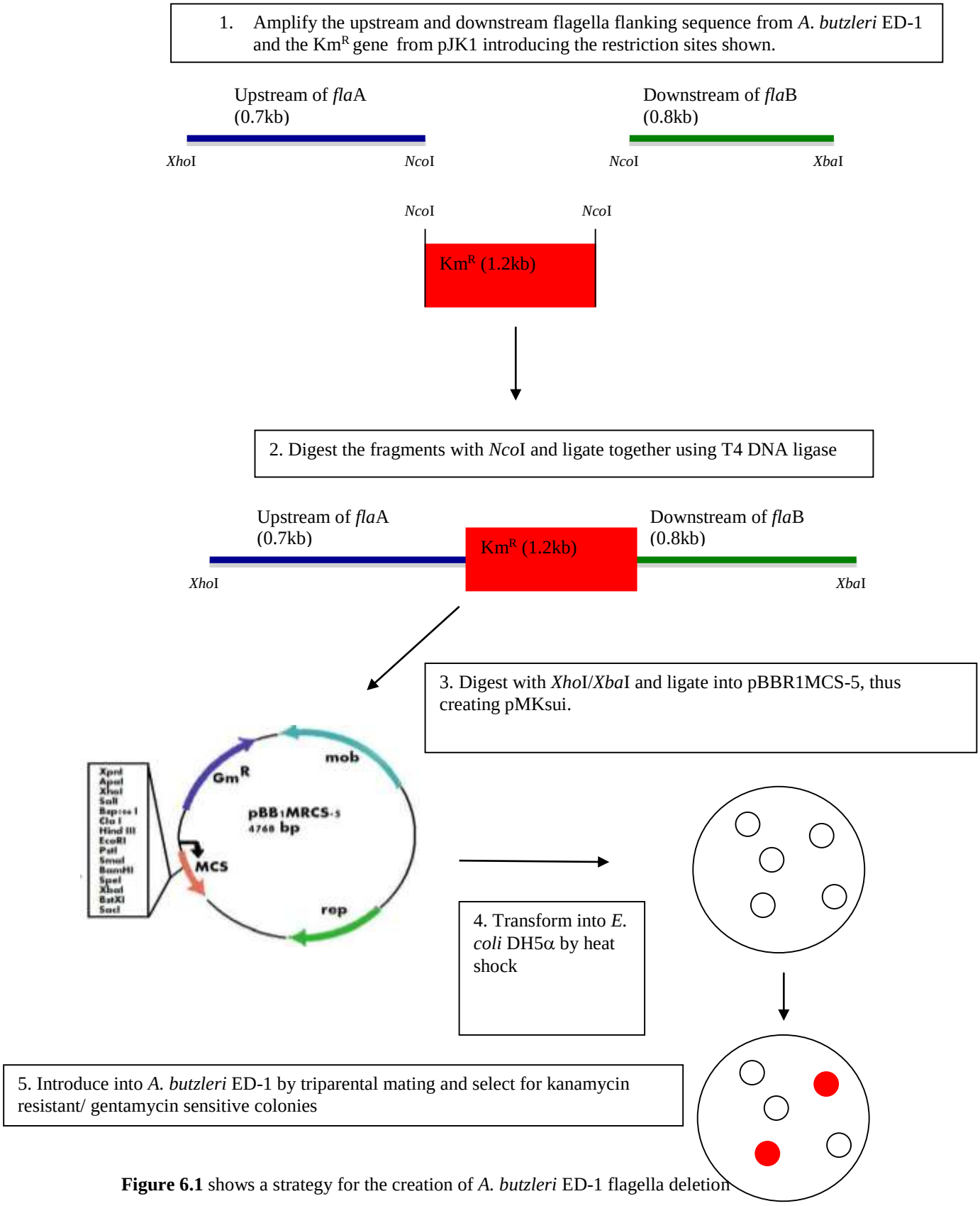


Figure 6.1 shows a strategy for the creation of *A. butzleri* ED-1 flagella deletion mutants using pBBR1MCS-5 (Kovach *et al.*, 1995) and the Km^R cassette from pJK1 (Kolmar 2009) to construct a suicide vector

6.1.2.2 Construction of a suicide vector using pWM1007, gentamycin resistance and crossover PCR (strategy 2)

The second strategy was based around the same principle i.e. an antibiotic resistance cassette flanked by upstream and downstream flagella flanking sequence although it was to be created by crossover PCR and cloned into the vector pWM1007, which was found not to replicate in *A. butzleri* ED-1 (see chapter 4). pWM1007 contained the *rk*2 *ori*T allowing it to be introduced to *A. butzleri* ED-1 by triparental mating using the helper plasmid pRK600. The antibiotic resistance cassette chosen was the gentamycin resistance cassette (Gm^R) from pBBR1MCS-5 (Kovach *et al*., 1995) as it was readily available. The constructed vector would be called pMKsui2. Figure 6.2 outlines the strategy for its creation.

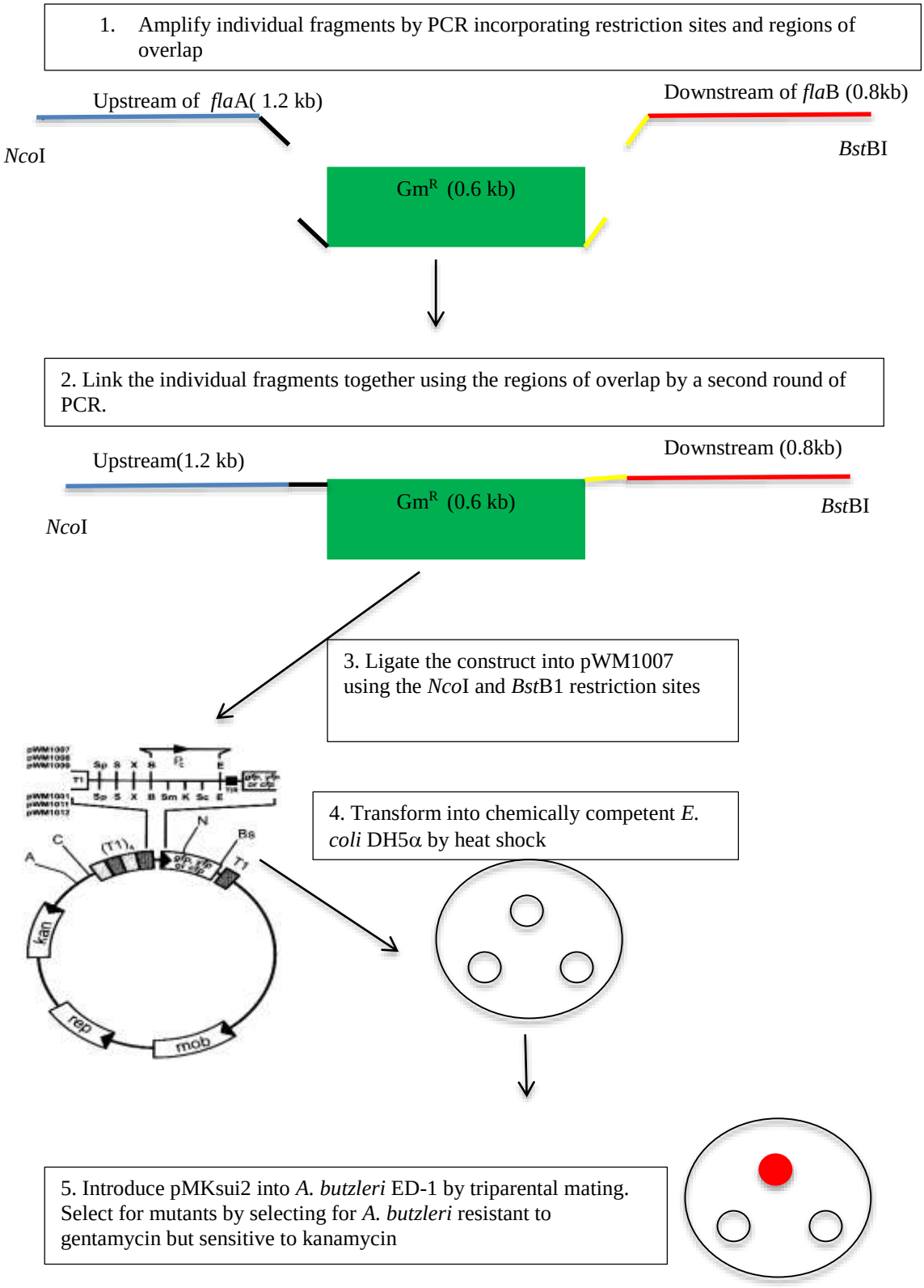


Figure 6.2 shows the construction of a suicide vector for the construction of *A. butzleri* ED-1 flagella deletion mutants using pWM1007 (Miller *et al.* , 2000), the Gm^R cassette from pBBR1MCS-5 (Kovach *et al.*, 1995) and crossover PCR

6.1.3 Aims and objectives

The aim of the study was create deletion mutants by either of the two strategies detailed above and use them to investigate the following.

- The role of flagella in *A. butzleri* ED-1 electrogenesis. This would be investigated by growing *A. butzleri* ED-1 mutants and wild type in half MFCs (see chapter 2 for set-up) and comparing the redox potential generated over a period of time. If flagella are important in electrogenesis then the half MFCs containing mutants would exhibit a less negative redox potential then half MFCs containing wild type *A. butzleri* ED-1
- The role of flagella in *A. butzleri* ED-1 biofilm formation. The mutant *A. butzleri* ED-1 would be tagged with GFP and CFP and subject to the same experiments used to investigate biofilm formation in wild type *A. butzleri* ED-1 (see chapter 4). If flagella are important in *A. butzleri* ED-1 biofilm formation then it would be expected to see mutant *A. butzleri* ED- less able to form biofilms then wild type *A. butzleri* ED-1.

6.2 Results

6.2.1 The attempted construction of a suicide vector for the creation of *A. butzleri* ED-1 *fla*A-*fla*B deletion mutants using strategy 1

6.2.1.1 Amplification of individual fragments by PCR

The first stage of creating pMKsui was to amplify the individual fragments by PCR. These fragments were obtained on the first round of PCR with no need for optimisation, the presence of the PCR product was confirmed by running a 1% (w/v) agarose gel which is shown in Figure 6.3.

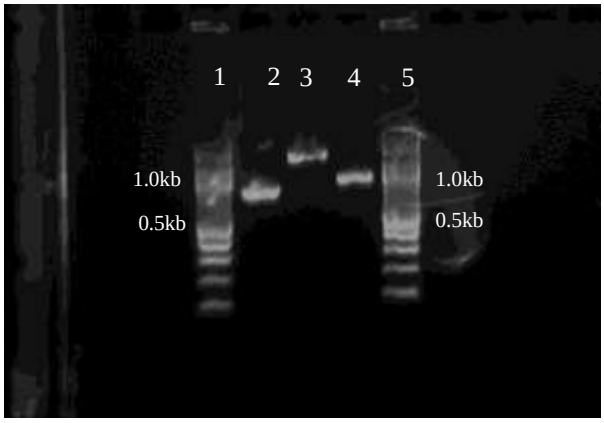


Figure 6.3 shows a 1% (w/v) agarose gel run in 1* TBE buffer at 120 V for 1 h. Lane 1) 100 bp ladder; lane 2) 0.7 kb upstream flagella flanking sequence ; lane 3) 1.2 kb Km^R cassette; lane 4) 0.8 kb downstream flagella flanking sequence; lane 5) 100 bp ladder. Each PCR reaction produced a single band of product after the first round of PCR with no need for additional optimisation. These products were then purified by PCR purification kit (See chapter 2 for more details of kit and protocol used).

Figure 6.3 showed that individual fragments were successfully amplified by PCR with upstream flanking sequence (0.7 kb), Km^R cassette (1.2 kb) and downstream flanking sequence (0.8 kb). A single band of product was observed for each reaction condition. The products were purified by Qiagen PCR clean-up kit (see chapter 2) then subject to digestion as described below.

6.2.1.2 Ligation of fragments and transformation into *E. coli*

With the successful amplification and purification of the individual PCR fragments the next stage was to digest the fragments with *Nco*I, ligate them together and sub-clone them into pBBR1MCS-5 before transforming them into *E. coli*.

Initially fragments were digested with *Nco*I for 3 h at 37 ◦C and dephosphorylated with Antarctic phosphatase (see chapter 2) for 15 min before heat inactivation of the enzyme and purification (by PCR clean-up) kit of the reaction mix. Recovery of the DNA was not verified by agarose gel electrophoresis nor quantified in any way. The pure fragments of DNA were joined together by incubation at room temperature overnight with T4 DNA ligase. Ligation of the construct into pBBR1MCS-5 was performed by digesting with *Xba*I and *Xho*I for 3 h at 37 ◦C. Following these steps the insert and vector were joined together as described above. The success of the

ligation at either was not verified by any means before proceeding with the transformation.

After ligation the ligation mix was transformed into chemically competent *E. coli* by heat shock as described in chapter 2. Successful recombinants were selected for using 50 μg/mL gentamycin and 50 μg/mL kanamycin; no successful transformants were recovered, although the cells were found to be competent by using a pJK1 control. A number of different approaches were taken as detailed in Table 6.1.

condition	Success
Fresh batch of competent cells	-
Single antibiotic selection	-
2 h recovery time	-
3 h recovery time	-
electroporation	-

Table 6.1 lists a number of different approaches to try and introduce supposed pMKsui into competent *E. coli* DH5a. It was decided to try with a freshly prepared batch of cells which yielded negative results. Using single antibiotic selection of 50 μg/mL gentamycin or 50 μg/mL kanamycin also met with failure. It was decided to extend the recovery time of each transformation to 2 h and 3 h although this met with failure. Finally it was decided to introduce the DNA to the cells by electroporation, which failed. The above conditions were also attempted with electro competent cells and no successful results were yielded. Controls used throughout was supercoiled pJK1 DNA, which yielded successful transformants.

The experiment was repeated with only the upstream and downstream fragments cloned into pBBR1MCS5 and clone the Km^R cassette into the holding vector once it had been successfully introduced into *E. coli*. The success of ligation was not verified before proceeding with transformation reaction.

Positive controls (using pJK1) yielded a relatively low number of colonies, with only 10-20 colonies being recovered (data not shown).

6.2.2 Creation of a suicide vector and deletion mutants by strategy 2

6.2.2.1 Amplification of individual fragments by PCR

The individual fragments required for the construction of pMKsui2 were first amplified by PCR. These fragments were upstream flagella flanking sequence, downstream flagella flanking sequence and the Gm^R cassette from pBBR1MCS-5 (Kovach *et al.*, 1995). The upstream flanking sequence and Gm^R cassette were amplified successfully in the first round of PCR without any further optimisation; although the downstream fragment required several different optimisation steps including a redesign of the reverse primer before the correct PCR product was successfully produced. The final conditions for PCR are given in chapter 2.

Images of the agarose gels for the successfully acquired downstream flagella fragments and the Gm^R cassette are shown in Figure 6. 4. An image of the upstream flagella fragment is not shown due to poor quality of the available images.

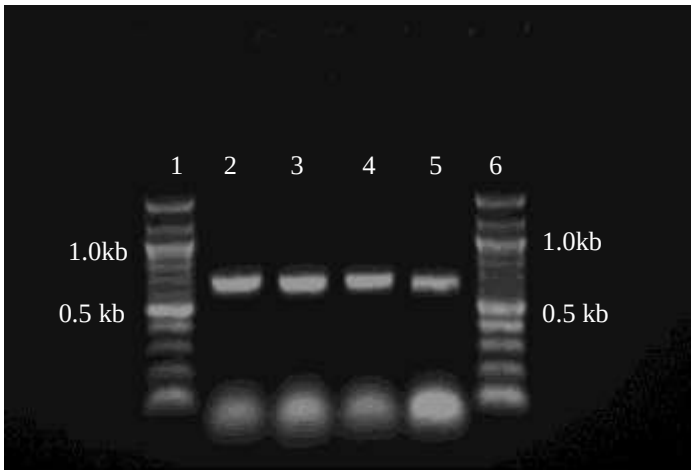


Figure 6.4 shows 1% (w/v) agarose gel run in 1* TBE buffer for 1h at 120 V. Lanes 1 and 6 contain 100 bp ladder. Lanes 2, 3 and 4 show the 0.8 kb flagella downstream fragment whereas lane 5 contains the 0.6 kb Gm^R from pBBR1MCS-5 (Kovach *et al*., 1995). The flagella downstream fragment took several rounds of PCR to acquire including one redesign of the reverse primer. The optimal conditions and primer are listed in chapter 2. The Gm^R cassette was acquired in the first round of PCR.

Figure 6.4 showed that individual fragments of Gm^R cassette (0.6 kb) and downstream flagella fragments (0.8 kb) were successfully obtained as single products. The upstream flagella fragment (1.2 kb) was also obtained as a single product.

6.3.2.2 Attempts at joining the fragments together by crossover PCR

Following the acquisition of the individual PCR fragments and their purification by DNA purification kit the next stop was to join the fragments together in the order upstream flagella fragment-Gm^R-downstream flagella fragment by crossover PCR. This method used the overlapping regions of sequence on the 3' end of the flagella upstream fragment, the 5' and 3' end of the Gm^R cassette and the 5' end of the flagella downstream fragment to align the fragments in the correct order and join them together by a PCR reaction using the forward primer for the flagella upstream fragment and the reverse primer for the flagella downstream fragment.

Multiple attempts at crossover PCR failed, despite use of chemicals such as DMSO and application of temperature gradients to determine the optimum annealing temperatures for the primers used.

6.3 Discussion

Neither strategy resulted in the successful creation of deletion mutants with neither strategy progressing beyond the amplification of individual fragments by PCR. There were several possible reasons for the failure of both strategies.

6.3.1 Lack of recovery of DNA by PCR clean up kit

Both strategy 1 and strategy 2 used a Qiagen PCR clean up kit (see section 2.2) to purify the individual fragments amplified by PCR. Therefore one of the most likely explanations for the failure of subsequent steps in both strategies was failure to recover DNA from the PCR clean-up kit. Due to time constraints recovery of DNA was not verified by agarose gel electrophoresis, therefore both strategies should be repeated with recovery of DNA verified by agarose gel electrophoresis before proceeding with the cloning strategy.

The most common reasons for the lack of recovery of DNA are incorrect application of buffer to the column membrane, use of a buffer other than the eluting buffer supplied with the kit or water or the lack of ethanol in the PE buffer. Therefore new reagents should be used with careful attention paid to their preparation and application when repeating the strategy. The lack of DNA would prevent successful completion of both strategies as it would mean lack of DNA for ligation and to serve as a template for crossover PCR.

6.3.2 Failure of transformation during strategy 1

The ligation mix was not successfully transformed into competent cells during strategy 1 with the only colonies recovered being positive controls. There were several possible reasons for the failure of transformation of the potential construct into *E. coli*.

The first reason for the failure of transformation was that the ligation was unsuccessful. Therefore fragments of linear DNA that would not replicate were being used. It is unknown whether the ligation was successful or not as the success of the ligation was not checked before proceeding with the transformation due to time constraints. Another alternative would be to repeat the cloning strategy using ligation independent cloning and see whether this yielded a more successful result.

The use of only an *Nco*I restriction site to ligate the Km^R cassette to both the upstream and downstream fragments could mean that the Km^R cassette aligned in the wrong orientation and did not express correctly. It could also mean the upstream and downstream fragments joined to each other as opposed to the kanamycin cassette. This is unlikely as single antibiotic selection would have recovered these clones. One way around this is to redesign the primers to introduce a greater variety of restriction sites into the individual fragments.

The competent cells were inefficient. This is a likely explanation as a relatively low number of colonies (approximately 10-20) were recovered for the control plasmids, when previously >100 colonies have been recovered. This was observed when a fresh batch of competent cells were prepared. The viability of the cells should be tested by transforming uncut vector alongside the ligation mix and another alternative would be to use commercially available competent cells.

Therefore the transformations should be repeated with several quality control steps implemented at each stage such as the verification of ligation and the viability of

competent cells tested. Alternative strategies such as ligation independent cloning and the use of commercially available competent cells should also be employed.

6.3.3 Failure of crossover PCR during strategy 2

As discussed in section 6.4.1 one reason for the failure of crossover PCR could be the lack of recovery of DNA from PCR purification meaning that there was no template to perform crossover PCR with. Therefore the template used for crossover PCR should be analysed by agarose gel electrophoresis.

A positive control step was not employed when performing crossover PCR therefore it is unknown whether the reagents used were viable. Therefore the crossover PCR reactions should be repeated with a suitable positive control in order to show whether the reagents were viable.

Another possible reason for the failure could be the highly similar sequences in overlapping sequences between flagella upstream and the Gm^R cassette and flagella downstream and the Gm^R cassette. The high degree of sequence homology could lead to miss alignment between the different fragments and the formation of secondary structures within the DNA which, due to the high G/C content of the regions of overlap would be hard to disrupt. Therefore the primers should be redesigned to include overlaps that have a lower G/C content and less similar sequences.

After multiple attempts to perform a crossover PCR it was decided to abandon the attempts and reconsider the strategy for use at a future date.

6.4 Conclusions

The failure of both strategies to create a suicide vector means that no further work on the role of flagella in electrogenesis and biofilm formation by *A. butzleri* ED-1 was possible.

In fact the only conclusion that can be drawn from these results is that the strategies employed were flawed and require revision and the implementation of several quality control steps before deletion mutants could be successfully constructed.

Chapter 7

Overall conclusions

Throughout the project a number of different conclusions about *A. butzleri* ED-1 and *Arcobacter* L were drawn.

7.1 *A. butzleri* ED-1 is capable of using a variety of short chain organic acids as carbon sources and the methylcitrate cycle plays a role in acetate metabolism

A series of growth experiments showed that *A. butzleri* ED- 1 was capable of using acetate, fumarate, glutamate, lactate, malate and succinate as its sole carbon source. This was predicted by the metabolic reconstruction (Ler 2009) and therefore the assertions of the metabolic reconstruction were deemed to correct.

In addition several enzymes of the methylcitrate cycle were found to be up-regulated when *A. butzleri* ED-1 was grown with an excess of acetate supplied. The metabolism of acetate via the methylcitrate cycle was predicted by the metabolic reconstruction (Ler 2009) and therefore it was assumed to be correct with regards to acetate metabolism.

Therefore it was concluded that *A. butzleri* ED-1 could use a wide range of short chain organic acids as its sole carbon source and that it metabolises acetate via the methylcitrate cycle.

7.2 The up-regulation of FlaA in *A. butzleri* ED-1 harvested from the anode is thought to be due to its role in biofilm formation

FlaA was found to be up regulated 2.4 fold in *A. butzleri* ED-1 harvested from the anode of a half MFC compared to *A. butzleri* ED-1 harvested from an aerobic planktonic culture. A variety of studies have shown that flagella are important in biofilm formation in *Campylobacter spp*. with flagellin proteins being up-regulated at the anode and mutants lacking flagellin being found to be deficient in their ability to form a biofilm (Joshua *et al*., 2006, Kalmokoff *et al*., 2006). Therefore it is likely that FlaA plays a role in biofilm formation in *A. butzleri* ED-1. In order to confirm this theory the ability of FlaA deletion mutants to form biofilm needs to be investigated.

It was thought that the up regulation of FlaA observed at the anode was due to the protein playing a role in extracellular electron transfer based on the altered morphology of flagella observed in SEM micrographs of *A. butzleri* ED-1 growing on the anode and the expression of flagella in *Geobacter sulfurreducens* KN400, a highly electrogenic variant of *Geobacter sulfurreducens* (Yi *et al*., 2009). However more recent studies showed that the flagella of KN400 were not responsible for its highly electrogenic nature (Butler *et al*., 2012) and without a proteomic comparison of an anodic and non-anodic *A. butzleri* ED-1 biofilm it is impossible to tell whether FlaA is up-regulated specifically in response to interaction with the anode.

Therefore it was concluded that the most likely reason for the up regulation of FlaA at the anode is due to its role in biofilm formation and not electron transport, although further studies are required to confirm this.

7.3 *A. butzleri* ED-1 possibly performs electrogenesis by a method distinct from that of *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1

Unlike *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1, *A. butzleri* ED-1 did not display up-regulated electron transport proteins and adhesins when harvested from the anode (Nevin *et al*., 2009, Rosembaum *et al*., 2012). Unlike these two bacteria it did not form a thick anodic biofilm and a high number of cells were observed in the planktonic phase of half MFCs. Therefore it was concluded that *A. butzleri* ED-1 performed electrogenesis by a method distinct from that established for *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1. Before establishing this is the case further study of *A. butzleri* ED-1 anodic biofilms are needed most importantly a proteomic comparison of anodic and non-anodic biofilms to more specifically show which proteins are up-regulated only in response to growth at the anode.

Based on this information one possible basis for the model of electrogenesis by *A. butzleri* ED-1 is that the bacterium performs electrogenesis by a combination of direct electron transfer to the anode by an unknown electron transporter protein

without the production of nanowire appendages (which means that the *A. butzleri* ED-1 biofilm can only be a few cells thick) and by mediator compounds, which would allow the cells to proliferate in high numbers in the planktonic phase. There is precedent for the combination of direct contact and mediator based methods of electrogenesis with studies showing that *Shewanella oneidensis* produces mediator compounds in addition to growing as an anodic biofilm and thus high numbers are observed in the planktonic phase of MFC cultures (Marsili *et al*., 2008). However more studies need to be performed to confirm whether or not *A. butzleri* ED-1 produces mediator compounds. It must be noted that this new model is highly speculative and further studies are required before any further details of it are elucidated.

Therefore based on the lack of up-regulation of adhesins, electron transporter proteins at the anode and a formation of a thin anodic biofilm it was concluded that it is possible that *A. butzleri* ED-1 performs an electrogenesis by a model distinct from that established for *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 but further study is required to establish details about this possibly new model.

7.4 *A. butzleri* ED-1 appears to be more capable of maintaining a stable biofilm than *Arcobacter* L

It was concluded that in the half MFC and micro aerobic cultures studies that *Arcobacter* L established a biofilm faster but was unable to maintain a stable biofilm

with *A. butzleri* ED-1 appearing to maintain a stable biofilm over a longer period of time. There were several possible reasons for this including *Arcobacter* L being less resistant to acidification of the biofilm that would occur over time and *Arcobacter* L being less suited to the anaerobic conditions that would develop in half MFCs over time and would therefore be less able to maintain a biofilm than *A. butzleri* ED-1. The fact that *A. butzleri* ED-1 was found to maintain a more stable biofilm that *Arcobacter* L is one possible reason for cultures of *A. butzleri* ED-1 displaying a more negative redox potential than *Arcobacter* L and thus being thought to be the more electrogenic of the two species.

However it must be noted that these conclusions are based on the results from a single set of cultures and therefore the experiments need to be repeated at least in triplicate to see whether the results are reproducible. Biofilm formation of the two species needs to be studied under a wider range of conditions including different pH and with more stringent anaerobic conditions before any conclusions can be drawn.

Therefore it was concluded that one possible reason for *A. butzleri* ED-1 being more electrogenic than *Arcobacter* L is that *A. butzleri* ED-1 is capable of forming a more stable anodic biofilm than *Arcobacter* L although further study is required to confirm this.

7.5 final summations

In summation the main conclusions drawn from this project were that

- *A. butzleri* ED-1 was capable of using a wide range of short chain organic acids as its sole carbon sources and that the predictions of the metabolic reconstruction were validated to be correct experimentally.
- The most likely reason for the up regulation of FlaA at the anode is due to it playing a role in attachment to the anode and biofilm formation as opposed to a role in electron transfer, however without a proteomic comparison of anodic and non-anodic *A. butzleri* ED-1 biofilms the exact role of FlaA at the anode remains unknown.
- The thin anodic biofilm (i.e. a few cells thick) and lack of up-regulation of electron transporter proteins and adhesins at the anode suggest that *A. butzleri* ED-1 is possibly performing electrogenesis in a fashion distinct from *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 both of which display up-regulation of these proteins at the anode and form thick anodic biofilms. One possibility is that *A. butzleri* ED-1 performs electrogenesis by a combination of direct electron transfer and use of mediator compounds. However without further proteomic and biofilm formation studies and further investigation into whether or not *A. butzleri* ED-1 produces mediator's details of this new model remain spare and highly speculative.
- One possible reason for cultures of *A. butzleri* ED-1 being more electrogenic than *Arcobacter* L is that *A. butzleri* ED-1 is more capable of maintaining a stable anodic biofilm. However without repetition of these experiments and

further study of *Arcobacter spp*. biofilm formation this reason is unconfirmed.

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Novel Electrochemically Active Bacterium Phylogenetically Related to *Arcobacter butzleri*, Isolated from a Microbial Fuel Cell[▽]†

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Exoelectrogenic bacteria are organisms that can transfer electrons to extracellular insoluble electron acceptors and have the potential to be used in devices such as microbial fuel cells (MFCs). Currently, exoelectrogens have been identified in the *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria*, as well as in the *Firmicutes* and *Acidobacteria*. Here, we describe use of culture-independent methods to identify two members of the genus *Arcobacter* in the *Epsilonproteobacteria* that are selectively enriched in an acetate-fed MFC. One of these organisms, *Arcobacter butzleri* strain ED-1, associates with the electrode and rapidly generates a strong electronegative potential as a pure culture when it is supplied with acetate. A mixed-community MFC in which ~90% of the population is comprised of the two *Arcobacter* species generates a maximal power density of 296 mW/liter. This demonstration of exoelectrogenesis by strain ED-1 is the first time that this property has been shown for members of this genus.

A microbial fuel cell (MFC) is a mimic of a biological system in which microorganisms transfer electrons from organic compounds to a conductive external electron acceptor under anaerobic conditions (6). In an MFC, the electron acceptor is provided by an artificial anode, which is connected to an electric circuit. Although the basic processes involved in the generation of electricity by bacteria have been known for many years, recent interest in MFC development has been stimulated by the need to find alternative, carbon-neutral sources of energy generation. MFCs are particularly useful for breakdown of organic matter in wastewater treatment plants, in which production of electricity as a by-product can be used to power the process or can be sold to offset the cost of operation (6). At present, although the key principles of MFC design and operation are well understood (19), the technical aspects and particularly the microbiological aspects (18) are still in development. Further optimization of the design and microbial composition of these devices is desirable as current MFCs achieve power densities of no more than 1,550 mW/liter (7), which limits their real-world applications (6).

The basic microbiological characteristics which influence the efficiency of an MFC are bacterial metabolism and bacterial electron transfer. Although most current MFCs perform optimally when they contain a mixed microbial community, some pure cultures that exhibit strong electrogenic activity in the MFC environment have been characterized (19). The electrogenic properties and some aspects of extracellular electron transfer have been defined for pure cultures of organisms such as *Geobacter sulfurreducens* (2, 3), *Escherichia coli* (27), *Shewanella putrefaciens* (15, 16), *Rhodoferax ferrireducens* (5), *Rhodopseudomonas palustris* DX-1 (40), and *Ochrobactrum anthropi* YZ-1 (41). The current list of confirmed exoelectrogens includes representatives of four of the five classes of *Proteobacteria* (only the *Epsilonproteobacteria* are not represented), as well as representatives of the *Firmicutes* and *Acidobacteria* (18). However, it is likely that novel electrogenic bacteria remain to be discovered.

The metabolic characteristics required for an electrogenic bacterium depend upon the specific application for which an MFC is used, because not all electrogenic bacteria are able to fully oxidize several substrates. For example, *Shewanella oneidensis* oxidizes lactate to acetate under anaerobic conditions, while *G. metallireducens* oxidizes acetate but not glucose (20). *R. ferrireducens* can oxidize acetate, lactate, and glucose but does not degrade ethanol, another common fermentation end product (11). For this reason, MFCs which are employed in wastewater treatment when complex compounds have to be degraded are often inoculated with a diverse microbial community (for example, methanogenic sludge [30]). Degradation of acetate is a key bacterial characteristic because acetate is a primary organic intermediate in the degradation of organic matter in anoxic aquatic sediments. Moreover, the ability to use artificial electron acceptors (anodic electrodes) provides bacteria such as *Geobacteraceae* with a competitive advantage over other microorganisms under such conditions. Analysis of the microbial community firmly attached to anodes harvesting electricity from a variety of sediments demonstrated that microorganisms in the family *Geobacteraceae* were highly enriched on these anodes (2, 35). Moreover, it was shown that an MFC initially inoculated with methanogenic sludge failed to consume acetate in the absence of anodic electrodes over a 1-year period (8).

Arcobacter spp. are inhabitants of human and animal hosts (14, 37) and also occur in various environments, including

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wastewater (24), surface water (21), seawater (9), and groundwater (32). *Arcobacter* spp. belong to the *Epsilonproteobacteria*, which includes pathogens (e.g., *Campylobacter jejuni* and *Helicobacter pylori*), opportunistic pathogens, and nonpathogenic environmental isolates (4). Typically, these bacteria have genomes with low G+C contents (27 to 30%), although some *Epsilonproteobacteria*, such as *Wolinella* spp. and *Campylobacter curvus*, have higher G+C contents. The environmental bacteria group into four clusters: *Nautiliales*, *Arcobacter*, *Sulfurospirillum*, and *Thiovulgaceae*. The genus *Arcobacter* comprises *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii*, and *Arcobacter cibarius*, all of which have been isolated from animals or food (particularly poultry), as well as *Arcobacter halophilus*, *Arcobacter nitrofigilis*, "*Candidatus* Arcobacter sulfidicus," and a number of species characterized so far only at the 16S rRNA gene level (4). A feature of both "*Ca*. Arcobacter sulfidicus" and *Arcobacter* sp. strain FWKBO is autotrophic metabolism under microaerobic conditions, in contrast to the heterotrophic growth of *A. butzleri*. Both of these organisms use oxidation of sulfide to sulfur and are obligate autotrophs. Some *Arcobacter* spp. may be capable of Mn and Fe reduction; isolates from Black Sea sediments (36) oxidized acetate in the presence of Mn oxide. This was the first evidence of Mn or Fe reduction in nitrate-reducing *Arcobacter* microaerophiles and nitrate reducers; previously, the only other epsilonproteobacterium identified with this ability was *Sulfurospirillum barnesii*. Thus, organisms related to *Arcobacter* comprise an ecologically significant new group of dissimilatory Fe- and Mn-reducing bacteria.

In the present study we isolated and characterized two strains phylogenetically related to *Arcobacter* spp. which are selectively enriched in an acetate-fed MFC. One of these strains, *A. butzleri* strain ED-1, which specifically associates with the MFC electrode, shows electrochemical activity when it is grown on acetate, and hence it is the first example of an exoelectrogenic epsilonproteobacterium.

MATERIALS AND METHODS

MFC. The MFC used in this work was a horizontal multielectrode bioelectrochemical reactor (8) in which immersed cathodes were replaced by semidry cathodes as described in patent application WO 2009/050513 A2 (V. V. Fedorovich, 23 April 2009). However, the structure of the liquid flow and the interelectrode distances were the same as those in the original design of this reactor. To avoid loss of biomass, the system was equipped with an external vessel and pump for liquid-phase recirculation. The total volume of the liquid phase was 1.5 liters.

Analysis and electrical measurements. The acetate concentration was determined using a Hewlett Packard gas chromatograph equipped with a glass column packed with Chromosorb 101 (80/100 mesh). The column, injector port, and flame ionization detector temperatures were set at 170°C, 180°C, and 190°C, respectively. Argon was used as a carrier gas. Before analysis, samples of influents and effluents were filtered using 0.45-μm membrane filters (ME 25; Schleicher & Schuell, Germany). Current and voltage were measured using a VersaSTATE 3 potentiostat (Princeton Applied Research, United States), and potentials were measured using an Ag/AgCl reference electrode.

MFC inoculation. The anodic zone of the cell was seeded with microorganisms from marine sediment from Cramond, Edinburgh, United Kingdom. The sediment was obtained at a depth of 3 m below the high-tide mark, but samples were obtained at low tide, when the sediment was exposed to the air, and at a temperature of ~10°C. Initially, 0.5 liter of marine sediment was diluted with 1.5 liters of a basal solution containing 0.05 M phosphate buffer (pH 5.7), 200 mg/liter $CaCl_2 \cdot 2H_2O$, 250 mg/liter $MgCl_2 \cdot 6H_2O$, and 500 mg/liter NH_4Cl; the low pH was used to select against methanogenic community members. Following decanting of the supernatant, basal medium that contained sodium acetate at a final concentration of 1.0 g chemical oxygen demand (COD)/liter was added. The fuel cell was filled with this final solution, the peristaltic pump was switched on, and the device was operated at room temperature (20°C). The hydraulic retention time of the liquid phase in the anodic zone of the MFC was 0.5 day. Sodium acetate in basal medium was added at approximately weekly intervals to maintain the total concentration at 1.0 g COD/liter.

Batch experiments in flasks. Flasks with a total volume of 500 ml were used for determination of electrogenic activity with and without an anodic electrode. In experiments in which the electrogenic activity of the MFC microbial community was investigated, 500 ml of the complete MFC liquid phase was added along with components of the basal medium at the standard concentrations. In experiments with pure cultures of *Arcobacter* spp., the flasks were filled with 500 ml of basal medium plus acetate, and a 2% inoculum of the relevant strain(s) grown microaerobically at 30°C in VanDamme (VD) medium (see below) was then added. When appropriate, sterilized reference (Ag/AgCl), counter (platinum), and working (carbon tissue) electrodes were introduced through sealed ports in the upper lid of the flask. The geometric surface of the working electrode was 120 cm^2. Flasks were incubated statically at room temperature (20°C).

Extraction of DNA from the MFC. Total community DNA was extracted from 100 μl of MFC supernatant, a 100-mg portion of the graphite electrode, or 100 mg of the original sediment inoculum for the MFC using a SoilMaster DNA extraction kit (Epicentre Biotechnologies) according to the manufacturer's instructions. DNA pellets were resuspended in 300 μl of sterile, distilled water.

16S rRNA gene amplification and sequencing. Almost full-length bacterial 16S rRNA gene fragments were amplified using primers complementary to positions 19 to 38 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1581 to 1541 (5′-AAGGAGGTGATCCAGCCGCA-3′) of the *E. coli* 16S rRNA gene as described previously by Fernández et al. (10). The PCR products were amplified using 50-μl reaction mixtures containing 10 μl of a community DNA sample or cells obtained directly from a plate colony, 0.5 μM each primer, 0.2 mM each deoxynucleoside triphosphate, 5 μl 10× *Taq* buffer (final concentration of $MgCl_2$, 1.5 mM), and 0.25 U of *Taq* DNA polymerase (Roche Applied Science). The PCR conditions were those employed by Fernández et al. (10), except that when amplification was done directly with cell colonies, an extra initial denaturation step (94°C for 10 min) was added prior to addition of the polymerase. PCR products were purified using a QIAquick PCR purification kit (Qiagen) prior to cloning or direct sequencing. Sequencing was performed with an ABI3730 capillary sequencer (The Gene Pool, School of Biological Sciences, University of Edinburgh) using the primers that were used for the initial PCR amplification for direct sequencing of PCR products or primers M13 forward (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse (5′-AACAGCTATGACCATG-3′) for fragments cloned into pCR2.1 (see below). 16S rRNA gene sequences were queried against the GenBank database using BLAST-N and were aligned with 16S rRNA gene sequences of related organisms using CLUSTAL-W. A neighbor-joining tree based on the alignment was constructed using the program MEGA4 (34). Confidence estimates for the branches in this tree were obtained by bootstrap resampling analysis with 1,000 replicates.

RFLP and DGGE analyses. Purified 16S rRNA gene PCR products (~1,500 bp) were digested with HaeIII and HhaI (New England Biolabs) and electrophoresed on 3.5% (wt/vol) MetaPhor agarose-Tris-borate-EDTA gels (Lonza Corporation) at 4 V/cm and 4°C for 17 h. Restriction fragment length polymorphism (RFLP) fragments for cloning were first separated on standard (0.8%) Tris-borate-EDTA-agarose gels and then extracted from these gels using a QIAquick gel purification kit (Qiagen). For denaturing gradient gel electrophoresis (DGGE), nested primers 341F-GC (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAGCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) were used to amplify the V3 variable region of the bacterial 16S rRNA gene from 1 μl of purified 1,500-bp first-round PCR product as described by Muyzer et al. (25). The PCR conditions used were initial denaturation at 95°C for 5 min, followed by 28 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1.5 min and then final extension at 72°C for 10 min. Ten-microliter samples of the extension products were loaded onto DGGE gels (22) containing a linear 30 to 70% denaturant gradient (100% denaturant was defined as 7 M urea [42%, wt/vol] and 40% [wt/vol] formamide). Electrophoresis was carried out with a DCode universal mutation detection system (Bio-Rad, Hertfordshire, United Kingdom) in 7 liters of 1× Tris-acetate-EDTA buffer at a constant temperature of 60°C for 960 min at 75 V. Silver staining of gels was carried out as described by Nicol et al. (26).

Cloning of 16S rRNA gene fragments. Purified fragments of amplified 16S rRNA genes obtained from RFLP gels or directly from PCRs were cloned into pCR2.1 using a TA cloning kit and chemically competent *E. coli* One Shot

INVαF′ (Invitrogen) according to the manufacturer's instructions. For fragments of restriction digests and PCR products which were not processed immediately, A tails were added to the DNA fragments using *Taq* polymerase prior to cloning. Plasmid DNA was isolated from positive clones using a QIAprep spin miniprep kit (Qiagen), and the presence of the insert that was the correct size was verified by digestion with EcoRI (New England Biolabs) prior to DNA sequencing.

Enumeration of bacteria from the MFC. Counting of viable bacteria was carried out using VD medium containing the following components (per liter): special peptone (Oxoid LP0072), 10 g; Lab Lemco powder (Oxoid L29), 5 g; yeast extract (Oxoid L21), 5 g; sodium glutamate, 2 g; sodium succinate, 2 g; $MgCl_2$, 1 g; and agar, 16 g. The *Arcobacter* selective medium (Houf-CNT) consisted of VD medium supplemented with 5% defibrinated horse blood and the following antibiotics: cefoperazone (16 μg/ml), novobiocin (32 μg/ml), and trimethoprim (64 μg/ml). Plates were incubated at 30°C either in an incubator, in gas jars under 5% (vol/vol) O_2-10% (vol/vol) CO_2, or in an anaerobic cabinet.

Bacteria attached to the carbon electrode were released by sonication. A 2-cm-by-2-cm piece of carbon cloth was cut out and put in a universal bottle with 10 ml of phosphate-buffered saline (PBS). Loosely attached bacteria were released by shaking the bottle at 150 rpm at room temperature for 10 to 20 min. The supernatant was removed, and the cloth was washed with 10 ml of PBS. Firmly attached cells were released by sonication in 10 ml of fresh PBS using a Heat Systems XL series sonicator at power setting 3 (amplitude, 15 to 20%) three times for 30 s. To enumerate bacteria, 10-fold serial dilutions in PBS were prepared, and 5-μl aliquots were plated onto both VD medium and Houf-CNT as described above.

Transmission and scanning electron microscopy. For transmission electron microscopy, *A. butzleri* ED-1 and *Arcobacter* sp. liquid phase, here and after referred to as *Arcobacter*-L, were grown in VD medium, washed twice in 1× PBS buffer, once in 0.5× PBS buffer, and once in sterile deionized water, and resuspended in sterile deionized water. The suspensions were spotted onto Formvar carbon-coated copper grids, air dried, and examined using a Philips CM120 BioTwin transmission electron microscope at an accelerating voltage of 70 kV. For scanning electron microscopy of aerobically grown ED-1, 1 ml of cells was grown overnight at 30°C in VD medium, washed in 1× PBS, resuspended in 200 μl of 1× PBS, and fixed with an equal volume of 3% glutaraldehyde; 20 μl of the suspension was then spotted onto a poly-L-lysine-coated coverslip. The samples were washed with 1× PBS, 0.5 × PBS, and deionized water and then sequentially dehydrated with three changes of 100% ethanol. Dehydrated samples were dried using a critical point dryer, sputter coated with Au/Pd alloy, and visualized with a BioSEM scanning electron microscope at an accelerating voltage of 5 kV. Square samples of a carbon electrode with attached ED-1 cells were fixed with 3% glutaraldehyde and then washed, dried, and visualized as described above.

Biochemical and growth characterization of *Arcobacter* spp. Biochemical properties of the isolated *Arcobacter* strains were determined using the API Campy strip identification system (BioMérieux, France) according to the manufacturer's instructions. Antibiotic resistance was assessed using the MASTRING-S antibiotic disk system (MAST Group Ltd., United Kingdom). To assess growth on particular carbon sources, a 2% inoculum of the relevant strain grown microaerobically at 30°C in VD medium was added to 10 ml of basal medium containing 1 g/liter of a carbon source, and the culture was incubated microaerobically at 30°C for 108 h. The optical density at 600 nm (OD_{600}) was determined at the beginning and at the end of the experiment. Growth rates of the *Arcobacter* strains on acetate were measured by adding 2% inocula grown microaerobically at 30°C in VD medium to 900-μl aliquots of basal medium with 1 g/liter acetate in a 48-well plate and incubating the plate microaerobically at room temperature (20°C).The OD_{600} was determined every 12 min for 45 h with a plate reader, and the means of the readings for five independent wells of each strain were used to generate the growth curves.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of *A. butzleri* ED-1 and *Arcobacter*-L determined in this study have been deposited in the GenBank database under accession numbers FJ968634 and FJ968635, respectively. The 16S rRNA gene sequences of other species cultured from the MFC have been deposited under accession numbers FJ968636 to FJ968639.

RESULTS

Acetate consumption and generation of potential in the MFC. Following inoculation with marine sediment obtained from Cramond, Edinburgh, the MFC was operated in recirculation mode while it was provided with an acetate minimal medium containing $CaCl_2$, $MgCl_2$, and NH_4Cl (see Materials and Methods). During continuous operation of the MFC, acetate was added to bring the concentration to the starting concentration (1.0 g COD/liter) at approximately weekly intervals. After 3 months of continuous operation, the maximal power density achieved by the MFC was 296 mW/liter. This level of power density was achieved largely as a result of the relatively low internal resistance (9 ± 0.5 Ω) of our device. To test the acetotrophic and electrogenic activity of the mature MFC microbial community, samples of the MFC liquid phase were taken and used to measure acetate consumption in flasks with a carbon anode and in flasks without a carbon anode. When an anode was present, acetate in the medium was rapidly consumed, indicating that there was efficient acetate-degrading activity in the established microbial community in the MFC (Fig. 1). This consumption of substrate was accompanied by a decrease in the anodic potential to approximately −400 mV. However, the acetate-degrading activity was much lower in the absence of an anode, indicating that the presence of an external electron acceptor was essential for efficient metabolism of this carbon source by the microbial community.

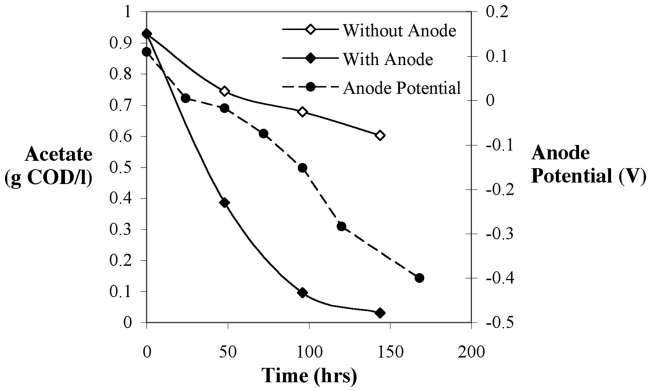

FIG. 1. Consumption of acetate (expressed in g COD/liter) in a flask containing the MFC liquid phase inoculated with a marine sediment microbial community following incubation with or without a carbon anode present. The development of the anodic potential over time with a carbon anode is also indicated.

Fingerprint analysis of bacterial diversity in the MFC. Samples of the liquid phase and graphite anode were taken from the operational, acetate-fed MFC following 4 weeks of continuous operation. Total community DNA was extracted from these samples and from the original marine sediment inoculum, and bacterial 16S rRNA gene fragments were amplified from the extracted DNA and analyzed by the RFLP method (Fig. 2A). This analysis showed that the samples were dominated by a few major species (bright bands) but that not all of these species were present in both the initial sediment and the MFC samples. In particular, a strong band at around 330 bp (Fig. 2A) was observed for the MFC samples but not for the environmental sample. As this band presumably represents a species which was selected for in the MFC environment, it was extracted from the electrode DNA sample in the gel, cloned, and sequenced. The sequence of the resulting 328-bp 16S rRNA gene fragment showed 100% identity to the 16S rRNA gene sequence of *A. butzleri* strain RM4018, an organism known to be an opportunistic human pathogen (23). Although

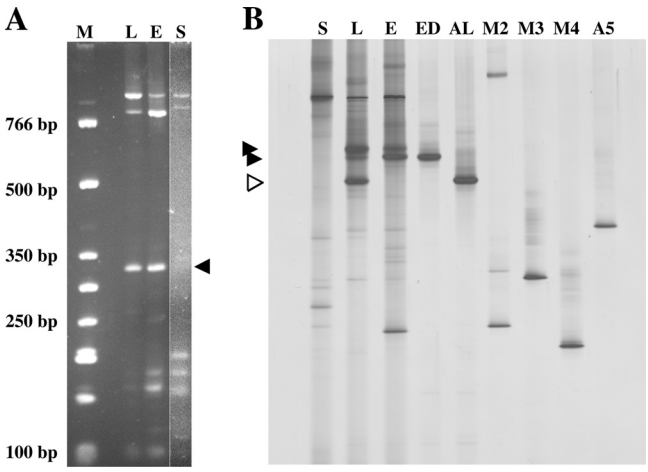

FIG. 2. (A) RFLP analysis of amplified, full-length bacterial 16S rRNA gene fragments from the MFC liquid phase (lane L), the carbon anode (lane E), and the original sediment inoculum (lane S). Lane S was overexposed to show the presence of faint bands and the absence of the 330-bp band specific to the MFC samples (arrowhead). Lane M contained size markers. (B) DGGE analysis of the amplified V3 variable region of bacterial 16S rRNA genes from the original sediment inoculum (lane S), the MFC liquid phase (lane L), and the carbon anode (lane E). Also shown are the DGGE profiles obtained for the cloned 16S rRNA genes of strain ED-1 (filled arrowheads) (lane ED) (amplification of 16S rRNA genes from genomic DNA of this species also gave rise to the upper band of the doublet) and the liquid-phase organism *Arcobacter*-L (lane AL) (open arrowhead). The gel also shows the DGGE profiles of 16S rRNA genes amplified from pure cultures of various species cultured from the MFC, including *Shewanella halitosis* (lane M2), *Acinetobacter* sp. (lane M3), *O. anthropi* (lane M4), and *Acinetobacter haeomlyticus* (lane A5) (see Table 2).

Arcobacter spp. have not previously been shown to be electrogenic, *Arcobacter* has been observed to be an unselected member of the community in a formate-enriched MFC (12). We also carried out DGGE fingerprint analysis of the V3 variable regions of bacterial 16S rRNA genes in the sediment and MFC samples, which was more sensitive to minor sequence variations in the 16S rRNA gene than RFLP analysis. The results (Fig. 2B) confirmed that the bacterial diversity in the mature MFC was quite different from that in the original sediment inoculum and also that there were differences between the electrode and liquid-phase samples. One prominent DGGE band for the liquid-phase sample (Fig. 2B) was not observed for the electrode sample, while a doublet of bands (Fig. 2B) was observed for both phases; none of these bands was detected when the initial sediment inoculum was examined.

Clone library analysis of bacterial diversity in the MFC. Small clone libraries (10 clones each) containing full-length 16S rRNA gene inserts were made using the same liquid- and electrode-derived community DNA samples that were used for RFLP analysis. Sequences from these clone libraries indicated that the electrode community was dominated by an RM4018-like *Arcobacter* strain (which we designated *A. butzleri* strain ED-1); 8 of 10 clones corresponded to this sequence (Table 1). However, analysis of the liquid-phase community revealed the presence of a different *Arcobacter* strain (*Arcobacter*-L). The two *Arcobacter*-like organisms share 95% sequence identity for 1,510 bp of the 16S rRNA gene (see Fig. S1 in the supplemental material). Again, *Arcobacter*-L dominated the liquid-phase community, and 8 of 10 clones corresponded to this organism (Table 1). The relatedness of the cloned 16S rRNA gene sequences of strain ED-1 and *Arcobacter*-L to 16S rRNA gene sequences of other members of the *Epsilonproteobacteria* is shown in Fig. 3. The 16S rRNA gene sequence of strain ED-1 was identical to that of strain RM4018, whose genome has been sequenced, and preliminary whole-genome sequencing of strain ED-1 (our unpublished data) showed that there was a high level of similarity and general colinearity of the two genomes. However, there were also significant genetic differences; for example, strain ED-1 lacked the sulfur oxidation and cytochrome *bd* oxidase genes.

The V3 regions of the strain ED-1 and *Arcobacter*-L 16S rRNA genes were amplified from the corresponding clones and analyzed alongside the mixed-community samples by DGGE (Fig. 2B). The ED-1 clone produced the lower band of the prominent doublet seen in both the electrode and liquid-phase communities, confirming that ED-1 is the main electrode-associated species in the MFC and indicating that this organism is also present in the liquid phase. DGGE of ED-1 genomic DNA-derived PCR products also produced the upper band of this doublet (data not shown), suggesting that the doublet is a PCR-DGGE artifact derived from the ED-1 16S rRNA gene sequence. The *Arcobacter*-L sequence corresponded to the additional major band found in the liquid-phase community, which again is consistent with its distribution in the clone libraries.

The remaining two clones obtained from the electrode and

TABLE 1. 16S rRNA gene clones obtained from the electrode and liquid-phase samples

Sample	Clone(s)	Closest database match	Accession no.	Length of overlap (nucleotides)	% Identity
Electrode	1–4, 6, 7, 9, 10	*A. butzleri* RM4018	CP000361	1,509	100
	5	Uncultured *Acinetobacter* sp. clone TCCC 11087[a]	EU567041	326	100
	8	*Shewanella* sp. strain S4[a]	FJ589031	310	99
Liquid phase	1, 3, 5–10	Uncultured *Arcobacter* sp. clone DS118	DQ234201	1,512	98
	2	*A. butzleri* RM4018[b]	CP000361	1,150	100
	4	Uncultured bacterium mle1-2 (*Bacteroides* sp.)[b]	AF280841	1,415	95

[a] Non-*A. butzleri* ED-1 clones obtained from the electrode sample were all chimeric with ED-1-derived sequences. The approximate length of the non-ED-1 portion of the sequence is indicated by the length of overlap.

[b] Non-*Arcobacter*-L clones obtained from the liquid sample were all chimeric with *Arcobacter*-L-derived sequences. The approximate length of the non-*Arcobacter*-L portion of the sequence is indicated by the length of overlap.

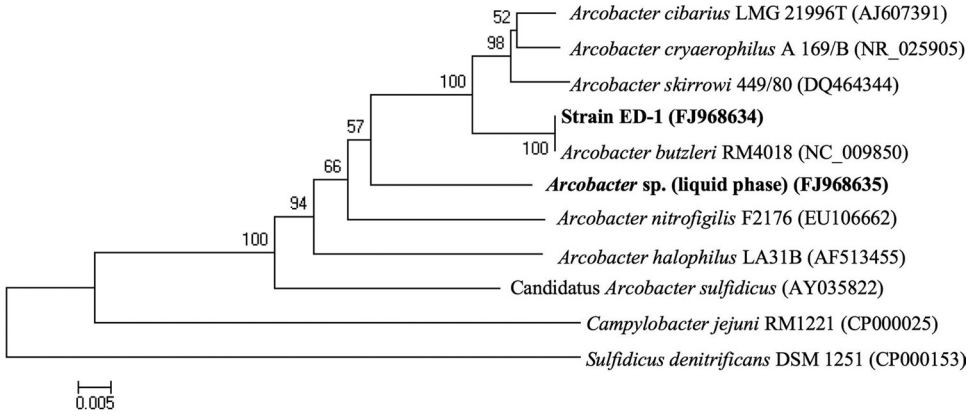

FIG. 3. Phylogenetic tree based on full-length 16S rRNA gene sequences for strain ED-1 and *Arcobacter*-L (bold type) and various other members of the *Epsilonproteobacteria*. Accession numbers for the sequences are indicated in parentheses. The tree was constructed using the neighbor-joining method; the bootstrap values at the nodes were calculated using 1,000 replicates. Bar = 0.5% sequence divergence.

liquid-phase samples were chimeric, a common feature of 16S rRNA gene-derived clone libraries, particularly when they are dominated by one sequence (38, 39). For the electrode sample, the chimeras consisted of sequences derived from an uncultured *Acinetobacter* species and *Shewanella* sp. strain S4 fused to the ED-1 sequence. One chimera from the liquid-phase sample consisted of an uncultured *Bacteroides* sequence fused to the *Arcobacter*-L sequence; the second chimera was a fusion between the 16S rRNA gene sequences of ED-1 and *Arcobacter*-L (Table 1).

Enumeration and isolation of bacteria from the MFC by plate culturing. To isolate species from the MFC as pure cultures, a liquid sample was taken from the MFC after it had been operated for 3 months. Bacteria from this sample were enumerated on both VD medium and Houf-CNT medium as described in Materials and Methods. Three different growth atmospheres were used: aerobic, microaerobic, and anaerobic. Aerobic cultivation was in air in a standard bacteriological incubator, microaerobic growth was in gas jars containing 5% (vol/vol) O_2 and 10% (vol/vol) CO_2, and anaerobic growth was in a miniMACS anaerobic workstation. Despite the low redox potential of the culture medium, the bacteria isolated from the medium were mainly aerophiles or aerotolerant organisms and microaerophiles rather than anaerobic bacteria. The numbers of bacteria isolated from VD medium were similar whether acetate (20 mM) or glucose (10 mM) was added as an additional carbon source. The viable counts were 4.6×10^7, 4.5×10^7, and 2.1×10^4 CFU/ml for aerobic, microaerobic, and anaerobic growth, respectively.

Single-colony purification and replating allowed isolation of both strain ED-1 and *Arcobacter*-L from the plates, and the 16S rRNA gene sequences of these organisms were determined to be consistent with those shown in Fig. S1 in the supplemental material by direct PCR amplification and sequencing from colonies. It was estimated by plate counting and microscopy that ~90% of the viable organisms in the sample were these species, confirming that they had been stably maintained in the MFC during its additional 2 months of operation. However, we also isolated two *Acinetobacter* spp. from the culture plates, one of which had a 16S rRNA gene sequence identical to that obtained in our previous clone analysis, as well as an additional *Shewanella*-like organism (which grew only microaerobically) and an *Ochrobactrum* species (Table 2). DGGE of the *Shewanella* (isolate M2) 16S rRNA gene indicated that it was not a major species in the MFC operated for 1 month but was detectable in the original sediment (Fig. 2B); it may have proliferated in the MFC during the additional 2 months of operation prior to culturing. While the *Ochrobactrum* sp. (isolate M4) was not detectable in any of the community samples based on the DGGE profile, the two *Acinetobacter* spp. (isolates M3 and A5) were detectable in both the MFC liquid-phase and electrode communities.

TABLE 2. 16S rRNA gene sequences of non-*Arcobacter* colonies isolated by plating from the liquid-phase sample

Plate	Isolate	Closest database match	Accession no.	Length of overlap (nucleotides)	% Identity
Aerobic	3	Uncultured *Acinetobacter* sp. clone VE12D01[a]	GQ179716	1,405	99
	4	*Ochrobactrum* sp. strain c261	FJ950617	655	100
	5	*Acinetobacter haemolyticus*	AY047216	1,421	99
Microaerobic	2	*Shewanella* sp. strain JC 19[b]	FM887036	1,342	99
	3	Uncultured *Acinetobacter* sp. clone VE12D01[a]	GQ179716	1,428	99
	4	*Ochrobactrum* sp. strain c261	FJ950617	655	100

[a] The sequence of aerobic isolate 3 and microaerobic isolate 3 (*Acinetobacter* sp.) is identical to that obtained for clone 5 from the electrode DNA sample (Table 1).

[b] The sequence of microaerobic isolate 2 (*Shewanella*) is only 91% identical in a 207-nucleotide overlap with that obtained for clone 7 from the electrode DNA sample (Table 1).

TABLE 3. Characteristics of strain ED-1, *Arcobacter*-L, and *A. butzleri* RM4018[a]

Characteristic	Strain ED-1	*Arcobacter*-L	*A. butzleri* RM4018[b]
Cell shape	Vibrioid	Vibrioid or small rod	Vibrioid
Motility	++	+	++
Microaerobic growth	++	++	++
API Campy profile	6 401 130	6 401 130	6 401 130
Alkaline phosphatase	+	+	+
Assimilation of:			
Glucose	−	−	−
Succinate	+	+	+
Acetate	+	+	+
Propionate	+	+	+
Malate	−	−	−
Citrate	−	−	−
Antibiotic resistance			
Ampicillin	R	R	R
Cefoperazone	R	R	R
Gentamicin	S	S	S
Kanamycin	S	S	S
Novobiocin	R	R	R
Streptomycin	S	S	S
Trimethoprim	R	R	R
Growth on C sources			
Acetate	+	±	−
Lactate	+	+	+
Succinate	+	±	ND
Fumarate	±	+	+
Glutamate	±	±	ND

[a] R, resistant; S, sensitive; ND, not determined.
[b] Antibiotic resistance and C source utilization data for *A. butzleri* RM4018 were obtained from reference 23.

Characterization of the two *Arcobacter* species. Microscopic analysis, biochemical tests, and antibiotic resistance profiles were used to differentiate the two *Arcobacter* species and compare them to *A. butzleri* RM4018. Transmission electron microscopy of cultures grown in VD medium showed that the larger, more vibrioid cells of strain ED-1 apparent when light microscopy was used (Table 3) may have been due to cell division that was more active than that of *Arcobacter*-L (Fig. 4A and B). Both strains appeared to have appendages when they were examined by transmission electron microscopy, and scanning electron microscopy of ED-1 cells (Fig. 4C) showed that they had lateral and branched filaments, as well as clear polar flagella. Scanning electron microscopy of ED-1 attached to the carbon electrode (Fig. 4D) showed no such appendages; further work is required to determine whether these structures are nanowires involved in extracellular electron transfer. The biochemical properties and antibiotic resistance profiles of these isolates were characterized using the API Campy system and Mastrings antibiotic disks, respectively. The API Campy numerical profiles for strain ED-1 and *Arcobacter*-L were identical. Both strains were able to metabolize succinate, propionate, and acetate but not malate, citrate, or glucose, and they also had similar antibiotic resistance profiles (Table 3). Growth on the MFC minimal medium supplemented with a variety of compounds as sole carbon sources was assessed microaerobically over a 108-h period (Table 3). The data showed that while

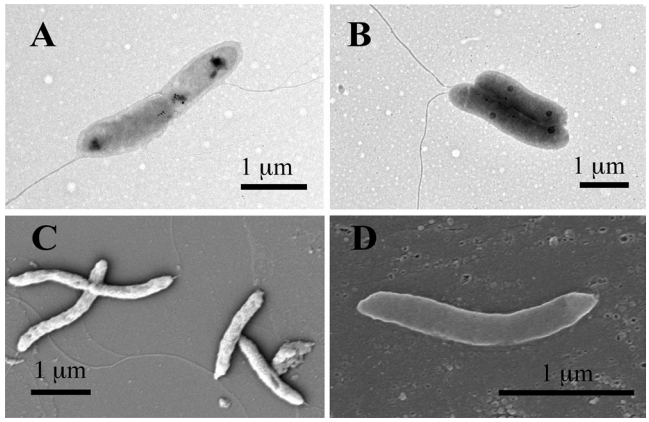

FIG. 4. (A and B) Transmission electron micrographs of strain ED-1 (A) and *Arcobacter*-L (B) following growth in VD medium. (C) Scanning electron micrograph of aerobically grown ED-1 cells, showing polar and branched appendages. (D) Scanning electron micrograph of an ED-1 cell attached to a carbon electrode following growth on acetate.

both *Arcobacter* strains grew well on lactate, strain ED-1, but not *Arcobacter*-L, showed significant growth on acetate; the reverse was true for growth on fumarate.

As acetate was the sole carbon source in the MFC from which these organisms were isolated, the growth of both *Arcobacter* strains on this compound was analyzed in more detail by growing multiple replicate cultures of each strain in a plate reader for 45 h (Fig. 5). The mean OD_{600}s obtained in this experiment showed that strain ED-1 grew rapidly on the acetate medium to an OD_{600} of >0.1, while *Arcobacter*-L showed much slower growth and the final OD_{600} was lower. This is consistent with the observation that ED-1 was the main acetotrophic species in the operational fuel cell and that ED-1, but not *Arcobacter*-L, was associated with the electrode. It is possible that *Arcobacter*-L persisted in the liquid phase of the MFC due to utilization of compounds produced by ED-1 and/or other members of the microbial community.

Electrogenic activity of strain ED-1. To test the electrogenic activity of a pure culture of strain ED-1, sterilized

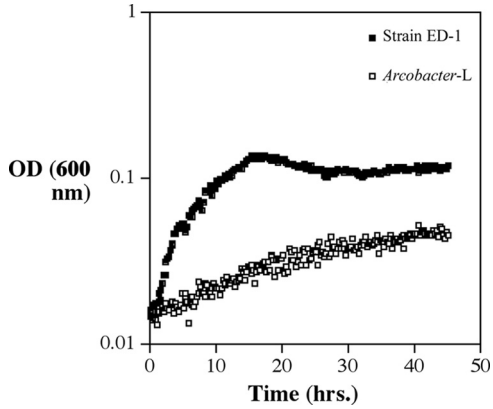

FIG. 5. Growth of strain ED-1 and *Arcobacter*-L on acetate minimal medium under microaerobic conditions in a microtiter plate at room temperature (20°C). The OD_{600} was measured every 12 min, and the values are the mean OD_{600}s for five independent culture wells.

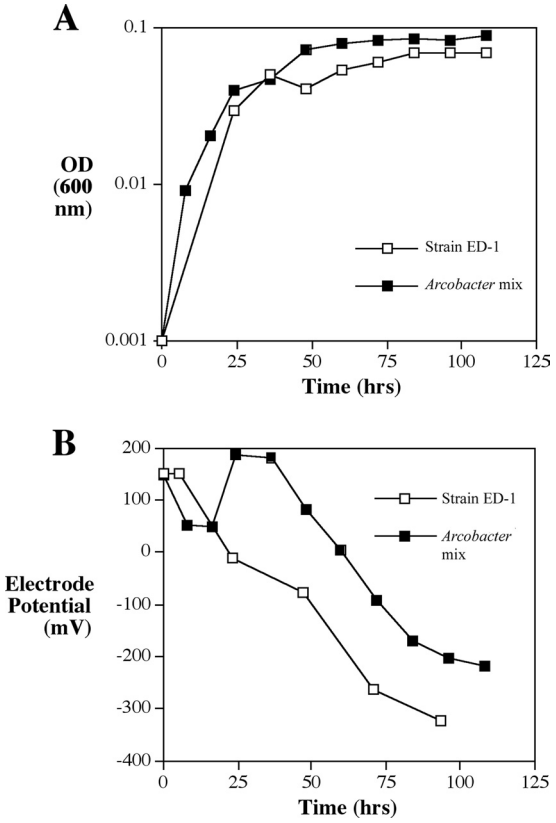

FIG. 6. (A) Growth of strain ED-1 and a culture started with a 1:1 inoculum of strain ED-1 and *Arcobacter*-L in acetate minimal medium in a sealed culture flask containing a sterilized, unconnected carbon electrode. (B) Electrode potentials at the carbon electrode in flasks containing the acetate-grown culture of strain ED-1 and the acetate-grown culture started with a 1:1 inoculum of strain ED-1 and *Arcobacter*-L shown in panel A.

flasks containing acetate minimal medium and a graphite electrode were inoculated with this organism or a 1:1 mixture of ED-1 and *Arcobacter*-L, sealed, and incubated at room temperature for 4 to 5 days. The growth of the bacteria and the anodic potential were monitored throughout this period (Fig. 6). Although the growth of the pure cultures on acetate medium under these conditions was similar to the growth of ED-1 in the absence of an electrode, significantly negative anodic potentials (−200 to −300 mV) were obtained in both ED-1 and mixed cultures within 4 days. Consistent with its superior growth on acetate and its association with the electrode, ED-1 was the dominant species in the mixed culture after 5 days, although the electrode potential obtained with the mixed culture was slightly less negative than that obtained with the ED-1 pure culture. These data confirm that *A. butzleri* strain ED-1 was the primary electrogenic species in the mature MFC.

DISCUSSION

Using a multielectrode horizontal MFC device inoculated with marine sediment, we isolated a novel *A. butzleri* strain which selectively proliferates on acetate in this environment and acts as a potent exoelectrogen. Transfer of the original sediment community to the MFC would have selected for an ability to utilize acetate and to transfer electrons to the anode, as well as an ability to proliferate at room temperature and at low pH. Our approach, which involved allowing the microbial community to adapt to this environment for 4 weeks and then characterizing the dominant microbial species present by culture-independent molecular methods, avoided having to use culture techniques to isolate electrogenic bacteria. Typically, exoelectrogens have been isolated from MFCs using agar plates containing soluble Fe(III) citrate (28), Fe(III) pyrophosphate (29), or insoluble Fe(III) oxide (13) as an electron acceptor, but these Fe(III) plating methods do not directly select for electricity-producing bacteria and may exclude electrogenic organisms which cannot respire using iron (41). Nutrient agar plates can also be used to isolate electrogens from MFCs (30), but the lack of selection with these methods allows nonelectrogenic bacteria to grow equally well. By first identifying the dominant species present in our MFC using culture-independent methods, we were able to isolate these species in pure culture on appropriate general rich (VD) and selective (Houf-CNT) media, along with other minor members of the microbial community. The validity of our approach was demonstrated by the observation that an estimated ~90% of the bacterial cells in the MFC as determined by plate counting and microscopy were cells of the species initially detected by our molecular techniques.

The mature, acetate-fed MFC community composed of ~90% *A. butzleri* strain ED-1 and *Arcobacter*-L cells proved to be highly efficient for generation of electricity. During continuous operation the fuel cell achieved a maximal power density of 296 mW/liter, which, although lower than the highest reported MFC power density (1,550 mW/liter for a bicarbonate buffer-containing fuel cell [7]), compares very favorably with the values reported previously for acetate-fed MFCs, 48 mW/liter (31) and 12.7 mW/liter (17). However, a portion of the difference can be attributed to electrode design and internal resistance rather than to the colonizing species. The same microbial community was able to consume acetate efficiently when an anode that acted as an artificial electron acceptor was present and to generate a strong electronegative potential (−400 mV) at the anode. However, although *Arcobacter*-L was the major planktonic component of this community, its role in acetate utilization and generation of electricity seems to be secondary to the role of the electrode-associated strain ED-1. Pure cultures of *Arcobacter*-L do not grow well on acetate, and a 1:1 mixture of *Arcobacter*-L and ED-1 inoculated into an electrode-containing flask and fed acetate rapidly becomes dominated by ED-1 and generates an electrode potential no more electronegative than that of a pure ED-1 culture. The persistence of *Arcobacter*-L in the MFC liquid-phase community may therefore be due to syntrophic interactions with other minor members of the microbial community and/or specific features of the MFC environment. In contrast, pure ED-1 cultures grow well on acetate and generate significant electronegative potentials in the presence of a carbon anode. *A. butzleri* strain ED-1 is therefore a potentially useful exoelectrogen for acetate-fed fuel cells.

It is of interest that, unlike the results for other enrichments of electrogenic, acetotrophic bacteria from marine sediments (2), no *Geobacter* spp. were isolated in our study. This may in

part be due to the low pH (pH 5.7) used for our MFC medium, compared to the higher pH (pH 6.8) generally used in *Geobacter*-containing fuel cells (3). Consistent with this hypothesis, in a formate-fed MFC in which *Arcobacter* sp. coexists with *Geobacter* sp. on the electrode, the pH of the medium is 7.0 (12). It is also possible that a low but nonzero level of oxygen in our system may have given the microaerophilic *Arcobacter* spp. an advantage over the strictly anaerobic *Geobacter* cells. Oxygen was not purged from the system following setup, and a small amount of oxygen would have been reintroduced during sampling. Consistent with this interpretation, the plate counts for liquid samples from the MFC were higher for aerophiles and microaerophiles than for anaerobes. The aerophiles may be instrumental in consuming oxygen in the system, thus maintaining the anode itself in a fully anaerobic environment and assisting electrogenesis.

As is typical for *Campylobacter* and *Arcobacter* spp. (23), strain ED-1 is unable to use sugars, such as glucose, as carbon sources. Analysis of the glycolytic pathway using the genome of *A. butzleri* RM4018 and RAST (1) indicated that the first step involving glucokinase is deficient but all other glycolytic enzymes catalyzing the formation of pyruvate are present. Our results differ slightly from those reported previously (23) in that strain ED-1 grew on acetate and propionate, both in microtiter plates and in an API Campy test kit. As a control we also tested *A. butzleri* RM4018 (= ATCC 12481) with the API Campy kit and found that this strain grew on both acetate and propionate (Table 3). However, the growth was more limited than the growth when the organism was grown in amino acid-containing media. In agreement with these data, we found that there was growth on lactate but not on citrate; the lack of growth on citrate may have been due to the absence of a citrate transporter. The inability to utilize glucose has also been found in other exoelectrogens, such as *G. sulfurreducens* (2), but strain ED-1 has an advantage over other species, such as *S. putrefaciens*, which cannot oxidize acetate under anaerobic conditions (33). Because acetate is the primary organic intermediate in a number of organic degradation pathways, its oxidation is an important step in accessing the full energetic potential of a variety of potential substrates, such as those found in wastewater samples. Thus, even if ED-1 is found not to be able to metabolize directly all the compounds present in a particular MFC substrate, it can potentially be a useful organism to add to MFCs utilizing such substrates in order to facilitate the efficient transfer of electrons from the acetate intermediate to the anode.

Our demonstration of electrogenic properties associated with the genus *Arcobacter* is novel and expands the range of microbial phyla which are known to act as exoelectrogens to include the *Epsilonproteobacteria*. There was one previous report of isolation of *Arcobacter* sp. from an MFC; Ha et al. (12) showed that a major DGGE band detected for DNA from the electrode of an MFC fed with acetate or formate gave rise to 16S rRNA gene clones with homology to *A. butzleri*, *A. skirrowii*, or *A. cibarius*. Surprisingly, the same DGGE band was equally prominent in the sediment inoculum used to initiate the MFC community, as were several other bands in the community profile. This suggests that in this system the selection pressure in the MFC environment was not as strong as that in our system, perhaps because the starting sample was derived from a sewage treatment plant rather than from natural sediments. Hence, *Arcobacter* spp. are not specifically enriched in this MFC environment; these authors speculated that *Arcobacter* spp. might persist in the MFC due to their preference for microaerobic environments and their ability to utilize formate, while they do not participate directly in electricity production (12). In contrast, our RFLP and DGGE analyses indicated that the two *Arcobacter* species that we isolated were not detectable in our original sediment inoculum but were the dominant species after 4 weeks of operation of the acetate-fed MFC. Therefore, strong selection for their enrichment in this environment was evident. In the case of strain ED-1, this was probably because it was able to utilize acetate in the medium as a carbon source and the graphite electrode as an electron acceptor more efficiently than other species in the initial inoculum and was also well adapted to other aspects of the MFC environment. Our community analysis also detected other species more typically associated with MFCs, such as *Shewanella* sp. (16) and *O. anthropi* (41) along with *Acinetobacter* sp., which were previously suggested to be involved in acetate consumption in the *Arcobacter*-containing MFC (12). However, DGGE analysis showed that, unlike the *Arcobacter* species, these additional species were not major members of the electrode-associated or liquid-phase communities in our MFC.

Arcobacter spp. include both environmental nonpathogens and opportunistic human pathogens. There is a growing list of opportunistic human-pathogenic bacteria which can act as exoelectrogens in an MFC, including *O. anthropi* YZ-1 (41) and *Pseudomonas aeruginosa* (30). *P. aeruginosa* can transfer electrons to an electrode via soluble mediators; the exocellular electron transfer mechanisms of *O. anthropi* YZ-1 remain to be determined. The closest relative of strain ED-1 is *A. butzleri* RM4018, which is considered an opportunistic pathogen. Although there is no current evidence of pathogenicity of strain ED-1, it has been speculated (41) that the production of exopolysaccharides by pathogenic species may help these organisms colonize MFC electrodes. *A. butzleri* RM4018 is known to produce lipooligosaccharides via a locus that is conserved among other *A. butzleri* strains (23); these molecules may be involved in the attachment of the environmental isolate strain ED-1 to the MFC electrode, but their role in the lifestyle of *Arcobacter* spp. has not been examined. Likewise, we need to understand more about electron transfer by strain ED-1. It will be interesting to examine whether the lateral appendages produced by ED-1 are involved in electron transfer by examining their ability to reduce silica ferrihydrite in vitro. Attempts to answer these questions and to determine the genetic basis of utilization of acetate and other carbon sources by ED-1 should also be aided by our ongoing genomic analysis of this strain.

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Quantitative proteomic analysis of the exoelectrogenic bacterium *Arcobacter butzleri* ED-1 reveals increased abundance of a flagellin protein under anaerobic growth on an insoluble electrode

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ABSTRACT

Exoelectrogens have the ability to generate electricity in mediator-less microbial fuel cells (MFCs) by extracellular electron transfer to the anode. We investigate the anode-specific responses of *Arcobacter butzleri* ED-1, the first identified exoelectrogenic *Epsilonproteobacterium*. iTRAQ and 2D-LC MS/MS driven proteomics were used to compare protein abundances in *A. butzleri* ED-1 when generating an electronegative potential (−225 mV) in an anaerobic half-cell — either growing as an electrogenic biofilm or suspended in the liquid medium - versus a microaerobic culture. This is the first quantitative proteomic study concentrating on growth of an exoelectrogen during current generation. From 720 proteins identified and quantified (soluble and insoluble sub-proteomes), statistical analysis reveals 75 differentially-expressed proteins. This dataset was enriched in proteins regulating energy and intermediary metabolism, electron and protein transport. Flagellin up-regulation was concomitant with electron transport in the anodic cells, while decreased abundance of a methyl-accepting chemotaxis protein suggested that flagella were involved in communication with the anode surface and electrogenesis, rather than motility. Two novel cytochromes potentially related to electron transport were up-regulated in anaerobic cultures. We demonstrate that employing an insoluble extracellular electron acceptor for anaerobic growth regulates multiple proteins involved in cell surface properties, electron transport and the methylcitrate cycle.

1. Introduction

In a microbial fuel cell (MFC), microorganisms oxidize organic matter and transfer electrons to the anode. These electrons flow through a circuit to the cathode, to combine with protons and a chemical catholyte, such as oxygen, resulting in the conversion of substrate into electricity [1]. Among their advantages over other energy generating technologies from organic matter, MFCs provide: direct conversion of substrate energy to electricity [1,2]; ambient temperature operation [3]; widespread applications for waste treatment and at locations lacking electrical infrastructures [4,5]. The ability of microorganisms to use diverse substrates as fuels makes MFCs suitable for renewable bioelectricity generation from biomass. However, their full-scale implementation is not straightforward and challenges, such as microbiological and technological understanding and economic

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viability, still remain. Research into all of these areas is required, in particular to understand how different microbial extracellular electron transfer mechanisms affect current output and, hence, MFC performance.

Many different exoelectrogenic bacteria that transfer electrons without the use of mediators to an MFC anode have been identified. For example, direct transfer has been widely studied in the dissimilatory metal reducing bacteria *Geobacter sulfurreducens* [6] and *Shewanella oneidensis* MR1 [7]. Several reviews discuss the putative mechanisms of direct transfer, typically involving at least a series of periplasmic and outer membrane complexes in these species [2,8]. In *S. oneidensis*, the apparent terminal cell-bound complex MtrCAB is involved in the transmembrane electron transfer mechanism [9]. It has also been suggested that electrically conductive pilus-like appendages called nanowires can facilitate electron transport between different microorganisms in biofilm communities [10]. A similar dependence on membrane-bound cytochromes, namely OmcZ, as well as the involvement of pilin nanofilaments, has been established to be essential for high current production in *G. sulfurreducens* [11]. Furthermore, such nanofilaments have been shown to have metallic-like conductivity in *G. sulfurreducens* biofilms within an MFC [12]. Indirect electron transfer employs electron shuttles or mediators to transport the electrons outside the cell to the electrode. Electron shuttles have been shown to be produced by some organisms as secondary metabolites, such as phenazines by *Pseudomonas aeruginosa* KRP1 [13] and flavins by *S. oneidensis* MR-1 and *Shewanella* sp. MR-4 [14]. Primary bacterial metabolites, such as H_2 and sulfur species, have also been found to conduct electrons to electrodes [15] and iron oxides [16], respectively. Although advances have been made in understanding electron transfer mechanisms of some exoelectrogenic bacteria, the mechanisms used by other and recently discovered electricity-producing organisms are still poorly understood. The current understanding of fundamental mechanisms is largely due to extensive bioelectrochemical and biophysical studies, and also genomics and some proteomics studies. Quantitative proteomics is still to play a major role in the characterization of the extracellular electron transfer pathways.

Arcobacter butzleri strain ED-1 was isolated from shallow marine sediments by selection in an acetate-fed MFC, and was shown to associate with the electrode and rapidly generate a strong electronegative potential as a pure culture when grown anaerobically on acetate [17]. The complete genomes of *A. butzleri* ED-1 and *Arcobacter*-L, have recently been sequenced [18]. *Arcobacter* spp. include both environmental nonpathogens and opportunistic human pathogens. The closest relative of strain ED-1 is *A. butzleri* RM4018, which is considered an opportunistic pathogen, and the ED-1 genome includes most of the putative virulence factors identified in RM4018 [18,19]. Although there is currently no direct evidence of pathogenicity of strain ED-1, it has been speculated that the production of exopolysaccharides by pathogenic species may help these organisms colonize MFC electrodes [20]. Lipooligosaccharides may be involved in the attachment of ED-1 to the electrode. Likewise, it will be interesting to examine whether the lateral appendages produced by ED-1 are involved in electron transfer [17]. To date, neither the role of lipooligosaccharides in the lifestyle of *Arcobacter* spp. nor the electron transfer mechanism in ED-1 has been fully examined.

We employ proteomics to understand more about *A. butzleri* ED-1 electrogenic behavior when operating under relevant current inducing conditions at the anode in a half-cell representation of an MFC. Two sub-sets of cells obtained from the anaerobic half-cell setup, cells attached as a biofilm to the anode (anode-associated or anode HC cells) and the planktonic cells in the half-cell chamber (anaerobic HC cells), were each compared with non-half-cell microaerobic planktonic (aerobic cells) culture. We examine both the soluble and insoluble sub-proteomes of each phenotype. iTRAQ (and other prominently applied silent isotope incorporation techniques) remains one of the most robust and easy-to-use techniques to be applied in quantitative proteomics [21]. Thus, we employ quantitative iTRAQ-based proteomics to gain a better understanding of the electron transfer mechanisms in exoelectrogenic bacteria during the process of shuttling electrons outside the cell membrane.

2. Materials and methods

2.1. Bacterial growth and strains

A. butzleri ED-1 cells were pre-prepared by culturing in 10 mL of Vandamme medium (10 g/L special peptone, 5 g/L Lab-Lemco powder, 5 g/L yeast extract, 5 g/L sodium chloride, 2 g/L sodium succinate hexahydrate, 2 g/L L-glutamic acid mono sodium salt, and 1 g/L magnesium chloride hexahydrate) and incubated at 30 °C under microaerobic (5% O_2, 10% CO_2 and 85% N_2) condition for 48 h [22]. For pre-culturing under microaerobic conditions, incubations were performed in a MACS-VA5000 microaerobic workstation (Don Whitley Scientific Limited). This cell culture was ready to inoculate either the anaerobic half-cells or microaerobic cultures, as described below. Microaerobic planktonic cultures of *A. butzleri* ED-1, i.e. under growth conditions where oxygen was the electron acceptor, were cultured in 200 mL volumes in 6×250 mL Duran bottles in the minimal medium described previously [17], and with 30 mM acetate. The medium was inoculated with a 4% v/v inoculum of *A. butzleri* ED-1 described above and incubated at 30 °C with 100 rpm shaking. The microaerobic planktonic cultures were incubated for 72 h until late exponential phase.

2.2. Half-cell setup and operation

In order to grow *A. butzleri* ED-1 in MFC-like conditions, 6 simple anodic half-cells were set up, which enabled us to obtain more cells to extract sufficient proteins for their iTRAQ quantitation. In essence, the half-cells are electrochemical chambers containing the bacterial cultures that generate the electricity, representing the negative terminal of a dual-chambered MFC, and allowing us to measure the voltage output of the anodic chamber. Half-cells have the advantage of being inexpensive and easy to replicate and sterilize, while retaining the characteristics of an MFC anode chamber. The half-cells used were constructed with a 250 mL Duran bottle and a modified lid introducing a carbon cloth electrode to serve as a working anode and an Ag/AgCl reference electrode (Hach Lange Ltd, UK). Supplementary Fig. 1 depicts the half-cells set up and location of the two sub-sets of anaerobic cells obtained for protein

extraction: biofilm or anode associated (anode HC) and anaerobic planktonic (anaerobic HC) cells. *A. butzleri* ED-1 was cultured in the half-cells using the same minimal medium used for microaerobic culture, with 30 mM acetate serving as the sole carbon source. Each half-cell was filled to the brim with minimal medium (250 mL) and inoculated with 4% v/v *A. butzleri* ED-1 pre-culture grown as described above. The half-cells were incubated at 30 °C under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) in a miniMACS anaerobic workstation (Don Whitley Scientific Limited) for 336 h until late exponential phase. After 96 h of growth, the cells were fed every 24 h by removing 20 mL of culture and replacing with 20 mL of fresh minimal medium with 30 mM acetate. To monitor the redox potential generated by the bacteria cultured within the half-cells, the potential difference between the carbon cloth electrode and the reference electrode was measured every 24 h using a Precision Gold voltmeter set on a 2-volt scale. Absorbance of the half-cell cultures was measured at 600 nm every 48 h. Selective plating of *A. butzleri* ED-1 in order to determine cell numbers was performed using Houf blood agar. Houf blood agar comprises Vandamme medium with 50 mL/L defibrillated horse blood and the following selective agents: 100 mg/L 5-flurouracil, 16 mg/L cefoperazone, 32 mg/L novobiocin, and 64 mg/L trimethoprim [23].

2.3. Protein extraction

Cells were harvested from non half-cell microaerobic planktonic cultures and the two sub-sets of anaerobic cells from the half-cell: anaerobic (planktonic) HC and anode (biofilm) HC samples (Supplementary Fig. 1 and Supplementary Information 1). Six anaerobic HC replicate samples were harvested from each half-cell, out of which four were used to extract the insoluble proteins for iTRAQ analysis 1, and the remaining two to extract the soluble proteins for iTRAQ analysis 2 (Supplementary Table 1). Similarly, six microaerobic cultures were used to extract soluble and insoluble proteins. As insufficient cells were harvested per anode for an iTRAQ sample, each of the two biological replicates for the anode HC samples used in iTRAQ analysis 3 and 4 resulted from the combination of pooling together cells from three half-cell anodes (6 half-cells in total). From each sample, insoluble and soluble proteins were extracted for quantitative proteomic analysis (Supplementary Fig. 2). Extraction of insoluble [24] and soluble [25] proteins were based on separate previously published protocols, with some modifications. In brief, cells were resuspended with 43 mM NaCl, 81 mM $MgSO_4$, 27 mM KCl buffer or 1 M TEAB (triethyloammoniumbicarbonate; pH 8.5), 0.12% SDS buffer for the insoluble and soluble protein extraction, respectively. The former buffer was detergent-free to achieve the isolation of the insoluble fraction (i.e. the fraction not dissolved in high salt-content buffers used for extractions), while the latter buffer used both TEAB and SDS to achieve more denaturation of proteins as well as membrane protein solubilization. Protein extraction of both fractions was carried out using an ultra sonicator (Sonifier 450, Branson) 10 times (alternately 30 s of sonication and 1 min on ice) at 80% duty cycle, followed by liquid nitrogen cracking and water bath sonication with ice for 15 min. Samples were then centrifuged at 2000 ×*g* for 5 min at 4 °C to remove unbroken cells and debris; the supernatant collected was then centrifuged at 100,000 ×*g* for 2 hrs or 21,000 ×*g* for 30 min for insoluble and soluble fractions, respectively. Before the total protein concentration was determined from both fractions using the RC-DC Protein Quantification Assay (Bio-Rad, UK), soluble and peripheral membrane proteins were removed from the insoluble fraction by washing the pellet with ammonium carbonate (pH 11) [26] and the pellet resuspended in 1 M TEAB, 0.12% SDS buffer.

2.4. iTRAQ analysis

iTRAQ labeling (one 4-plex and two 8-plex kits, one divided into two sets of 4 labels, were used; see Supplementary Table 1) was performed based on protocols detailed elsewhere [25] and our recommendations for a successful proteomic experiment [27]. Briefly, 100 (when comparing microaerobic vs anaerobic HC cells, due to sufficient extracted protein available) or 50 μg (when comparing microaerobic vs anode HC cells, limited extracted protein available) of protein from each condition was used. All samples were reduced, alkylated, digested, and labeled with appropriate iTRAQ reagents. Protein digestion was carried out using trypsin [25] or a combination of trypsin and chymotrypsin [24,26] for the soluble and insoluble proteins, respectively. For insoluble proteins, sodium deoxycholate (SDC) was added to a final concentration of 0.007% before trypsin digestion at a 1:10 (w/w) ratio overnight, and then a mixture of both trypsin and chymotrypsin with a 1:10 enzyme/protein ratio for the second day. Finally, digested peptides were combined prior to iTRAQ labeling (Supplementary Fig. 2). Four different iTRAQ experiments were performed for soluble and insoluble fractions from the three different conditions, with 2 or 4 independent biological replicates for each. Two biological replicates were used when not enough sample was available and was still acceptable for the purposes of this study, as we took account of this when calculating statistics (see below). iTRAQ reagents used as labels for each sample for each experiment are detailed in Supplementary Table 1. We used a combination of 4- and 8-plex iTRAQ because there were insufficient initial cell numbers to extract sufficient proteins to obtain 4 replicates for each type of sample.

After incubation at room temperature, labeled samples were combined before drying by vacuum concentration. First dimensional fractionation of samples using SCX (strong cation exchange) was performed on a BioLC HPLC (Dionex, UK) prior to second dimension RP (reverse phase)-LC MS/MS analysis. SCX fractionation was carried out using a PolySULFOETHYL-A Column (PolyLC, USA) with a 5 μm particle size, 10 cm length × 4.6 mm diameter and 200 Å pore size. The system was operated at 0.4 mL/min with an injection volume of 70 μL. Transfer and elution of peptides was achieved using binary mobile phase buffers A and B (buffer A: 10 mM KH_2PO_4 and 25% ACN at pH 3; buffer B: 10 mM KH_2PO_4, 25% ACN and 500 mM KCl, at pH 3). Separation was performed using a 60 min gradient: 5 min at 100% buffer A, followed by a linear ramp to 30% buffer B over 40 min, then 30% to 100% B over 5 min, and finally holding at 100% A for 5 min. A UV detector UVD170U calibrated at 214 nm and Chromeleon Software (Dionex, The Netherlands) were used to record the SCX chromatography. Labeled peptide fractions were collected every minute, and later dried by vacuum concentration.

2.5. Mass spectrometry analysis and data searching

Prior to tandem MS analysis, each fraction was first desalted using an UltraMicroSpin Column (The Nest Group, Southborough, MA, USA) following the manufacturer's protocol. Tandem mass spectrometry of LC-MS iTRAQ labeled samples was carried out on a QSTAR hybrid QTof (AB-SCIEX, Foster City, USA) and a maXis hybrid UHR-QToF (Bruker Daltonics, Coventry, UK), both coupled to an Ultimate 3000 nano-flow HPLC (Dionex), as reported previously [28]. All LC-MS iTRAQ samples were further desalted online using a 5 mm×300 μm ID LC-Packings C18 PepMap trap cartridge under 0.1% TFA and 3% ACN for 15 min, and eluted to a 15 cm, 75 μm ID LC-Packings C18 PepMap analytical column in 0.1% formic acid with an ACN gradient performed over a 90 min gradient (3–35% ACN). Details of MS and MS/MS level measurements for both instruments are provided in Supplementary Information 1.

Protein identification and quantification were mainly carried out as described by Ow et al. [28]. In brief, tandem MS data generated from an UHR-QToF were converted to MGF peak-lists via Compass Data Analysis v. 4.02 (Bruker Daltonics), while MS/MS raw data derived from the QStarXL were used to find peaks and convert to MGF format using the mascot.dll embedded script (V1.6) coupled with Analyst QS 1.1.1 (Applied Biosystems). MGF data from both instruments were interrogated (for identifications only) using an in-house cluster running the Phenyx algorithm [29] (binary version 2.6; Genebio, Geneva). iTRAQ MS/MS data were interrogated with the *A. butzleri* ED-1 predicted protein database obtained from the complete genome sequence [18], downloaded from http://www.ebi.ac.uk/ena/data/view/AP012047 on 2 September 2011 with 2158 protein entries. Details on the identification parameters and target-decoy database strategies [30] for the identification are provided in Supplementary Information 1 based on search criteria established elsewhere [24]. Only proteins satisfying a 1% global peptide false positive identifications or False Discovery Rate (FDR) [31], and observed with ≥2 peptides were considered for further quantitative analysis. The intensities of the iTRAQ reporters were automatically exported from all qualified (as above) spectra and processed as described by Pham et al. [25].

2.6. Analysis of iTRAQ quantification and statistical validation of differential expression

The analysis of iTRAQ ratio, with robust statistical tests (based on *p*-values) and their distributive estimations were all performed as described by Pham et al. [25] allowing confident identification of differentially expressed proteins. Protein quantifications were obtained by computing the geometric means of the reporters' intensities using an in house script developed in Mathematica (Wolfram). A global median correction and isotopic impurity correction was subsequently applied to every reporter in order to compensate for experimental and systematic errors. By using median-corrected intensities at the peptide level rather than at the protein level, as described elsewhere [25], we can achieve higher significance levels and distinguish between smaller fold changes when the evidence is overwhelming. *t*-test comparison between the reporter ions' intensities was carried out employing these corrected intensities. We tested whether the distribution of the values had a mean difference from zero, and reported the *p*-values associated with the said distribution, based on a threshold ($\propto$=5%; i.e. at the 95% confidence interval) for significance. If the protein is not differentially regulated, the relative intensities of the reporter ions are expected to follow a log-normal distribution with a mean of one. Calculating the *p*-value under this assumption, proteins have a significant change in abundance between anaerobic and microaerobic growth conditions when $p<0.05$ for every comparison of iTRAQ label pair. In other words, differential expression is ultimately based on the *p*-value, where any protein with a *p*-value below 5% is considered differentially expressed [27]. Since two or four biological replicates were available for each tested condition (Supplementary Table 1), differential expression was reported only if it was significant regardless of which replicate is chosen to perform the comparison. This was carried out for every protein. In the remainder of this paper, we refer to probability scores, defined as -log10 (*p*), to measure significance, and only report quantitative results that are statistically significant, thus requiring a score>1.3 [i.e. −log10($\propto$)]. All results are reported in the Supplementary Information 2 (iTRAQ 1 and 2) and 3 (iTRAQ 3 and 4), including the lists of quantified proteins and of regulated proteins.

3. Results and discussion

3.1. Bacterial growth in the half-cells and protein extraction

The first step toward making a comparison of the cellular proteins produced from anode -associated cells in a MFC with those produced in planktonic conditions was to grow cells of *A. butzleri* ED-1 both in a MFC set up and in flasks. Accordingly a pre-culture of *A. butzleri* ED-1, grown microaerobically in Vandamme medium, was used to inoculate replicate cultures in acetate minimal medium (see Materials and methods). These cultures were grown either microaerobically for 72 h in flasks or anaerobically in half-cells for 336 h, such that all cultures reached the late exponential growth phase. The anaerobic half-cell cultures contained a carbon cloth electrode (see Materials and Methods), allowing us to replicate the anode compartment of an MFC in a sterilisable, pure culture system. At the end of the appropriate growth period, ED-1 cells were harvested from the liquid phase of the microaerobic or anaerobic (half-cell chamber) growth vessels, and from the electrode biofilm of the half-cells; the latter two sets of cells are designated anaerobic HC and anode HC cells respectively. We then were able to compare protein abundances in cells that *use oxygen as a terminal electron acceptor* (*aerobic cells*) versus cells which can *use the carbon anode as an electron acceptor* (*anode HC cells*) (iTRAQ 3 for insoluble sub-proteome and iTRAQ 4 for the soluble), or versus cells that are *not provided with an exogenous electron acceptor* (*anaerobic HC cells*) (iTRAQ 1 for insoluble sub-proteome and iTRAQ 2 for the soluble).

We used 6 static half-cells to ensure reproducibility and to allow for a large enough quantity of protein to be extracted from anode HC (biofilm or anode associated) and anaerobic HC (anaerobic planktonic in half-cell) cells. Supplemental Fig. 3 shows the decrease in redox potential over the growth period, and indicates the reproducibility of the growth curves for each of the 6 cultures in the half-cells. The viable counts

were measured to assess the density and viability of the cells to attain average counts of $8.3\times10^8\pm0.6$ CFU mL^{-1} for anaerobic HC cells (Supplementary Table 2).

To mirror a useful MFC operational comparison, we allowed up to a 336-hour growth period to ensure the output of a low electrode potential (−225±40 mV) and sufficient anaerobic HC cells per half-cell for subsequent analysis (488±75 mg wet weight). Low anode HC cell amounts were harvested per anode (148±35 mg wet weight), hence three harvested anode HC cell samples were pooled together resulting in two biological replicate pools for protein extraction. We found that a combination of pulsed sonication, liquid nitrogen cracking and ice-water sonication in the specific extraction buffer (depending on the desired sub-proteome fraction) produced the most consistent and abundant protein preparations (Supplemental Fig. 4).

3.2. *Data mining: identification and quantification of differential protein abundance*

The method used to quantitate the differential abundance of bacterial proteins in the half cells compared to bacteria grown in flasks was iTRAQ, as it has major advantages over traditional gel-based quantitative approaches, not least in reproducibility, since it produces a relative quantification for each identified peptide [32]. This section describes the quantitation methods used, the number of proteins detected by iTRAQ, and the distribution of proteins between soluble and insoluble fractions, prior to analysis of which proteins were significantly associated with growth on the anode.

The relative quantification of proteins from the anaerobic cells taken from the half-cells, these being either anode associated (anode HC) or anaerobic planktonic (anaerobic HC) cells, versus non-half-cell microaerobically grown cells was carried out using two sets of 8-plex and one set of 4-plex iTRAQ labels. These iTRAQ sets were used to carry out four different analyses to include the soluble and insoluble sub-proteomes for each pair of compared phenotypes (Supplementary Table 1). As described in the methods, MS/MS data interpretation was performed using Phenyx peptide identification, and a quantitative data analysis algorithm using in-house developed Mathematica scripts.

Using Phenyx and our robust in-house statistical approach, we detected 3762 and 5582 peptide spectral matches (PSM) from the anaerobic HC vs microaerobic cells (iTRAQ 1 for insoluble sub-proteome and iTRAQ 2 for the soluble, respectively), corresponding to 169 and 233 unique identified proteins (402 in total) for each sub-proteome (see sheets 3 and 4 in Supplementary Information 2). We also detected 4656 and 8567 PSMs from the anode HC vs microaerobic cells (iTRAQ 3 for insoluble sub-proteome and iTRAQ 4 for the soluble, respectively), corresponding to 115 and 203 unique identified proteins (318 in total) for each sub-proteome (see sheets 3 and 4 in Supplementary Information 3).

Combined datasets from the insoluble and soluble sub-proteomes of anaerobic HC vs microaerobic planktonic cells comparison (iTRAQs 1 and 2, respectively) identified a total of 280 unique proteins, of which 122 are common to both sub-proteomes, while 47 and 111 were only identified in the insoluble and soluble sub-proteomes, respectively. Combined datasets from the insoluble and soluble sub-proteomes of anode HC vs microaerobic planktonic cells comparison (iTRAQs 3 and 4, respectively) identified a total of 211 unique proteins, of which 107 are common to both sub-proteomes, while 8 and 96 were only identified in the insoluble and soluble sub-proteomes, respectively (Supplemental Fig. 5).

Localization of identified unique proteins was also predicted. As a result, 61, 53, 22 and 54 predicted membrane proteins (including cytoplasmic membrane, outer membrane and periplasmic proteins; see Fig. 1) were identified by iTRAQ sets 1 to 4, respectively. From Fig. 1, 39 and 19 membrane proteins were common to iTRAQs 1 and 2, and iTRAQs 3 and 4, respectively. A high proportion of predicted cytoplasmic proteins was found in the insoluble fractions (46% and 70% for iTRAQ 1 and 3, respectively), many of which were ribosomal proteins (cytoplasmic proteins, see sheets 3 in Supplementary Information 2 and 3) possibly due to the attachment of these (nominally cytoplasmic) proteins to the membrane via nascent polypeptide chains [24,33]. Interestingly, more inner membrane proteins were found in all iTRAQ experiments compared to outer membrane proteins. A third of membrane and cytosolic proteins were found in common in merged datasets (30% for iTRAQs 1 and 2; 34% for iTRAQs 3 and 4), indicating that SDS used for protein extractions increased solubility of membrane proteins. It is clear that separate measurement of the insoluble and soluble sub-proteomes increased the total number of identified and quantified membrane and cytoplasmic proteins.

While the analysis identified only 10–13% of the total proteins encoded by the genome, the distribution of proteins detected showed that proteins were recovered from different subcellular fractions without bias. The reported detection sensitivity for prokaryotic proteomes using labeling and label free expression profiling combined with LC/MS varies in the range 9–80% [34,35]. For 2D-PAGE coupled with LC/MS, sensitivities of 10% have been reported both for *Xylella fastidiosa* and *Staphylococcus aureus* [36,37]. Despite the thin *A. butzleri* biofilms observed microscopically, the results from Fig. 1 show that numbers of proteins identified from the anode were of the same order as from planktonic cells, indicating sufficient protein was recovered for analysis. The number of proteins induced will depend upon factors such as environmental conditions, growth phase etc. as well as the proteome analysis method used. In a recent study [36] on *X. fastidiosa* growing planktonically and in biofilm conditions respectively, 387 and 456 proteins were detected from a genome complement of 2832 predicted proteins with 144 differentially regulated under biofilm conditions, a detection ratio similar to that reported here.

A robust statistical method was used to determine protein abundance changes, resulting in 22, 20, 4, and 29 differentially expressed proteins for iTRAQs 1, 2, 3 and 4, respectively (Tables 1 and 2). The localization of altered-abundance proteins is shown in Fig. 2. In combined iTRAQs 1 and 2, 44% were membrane related proteins and 41% cytosolic proteins, and in iTRAQs 3 and 4, 27% were membrane related and 61% cytosolic proteins. Eight altered-abundance proteins were common to iTRAQs 1 and 2, while no altered-abundance proteins common to iTRAQs 3 and 4 were found (Supplemental Fig. 6). It is also noted that the results likely present underestimation when calculating the protein differential expression fold changes. This underestimation results from compression of the iTRAQ ratio, such that a lower fold change is effectively

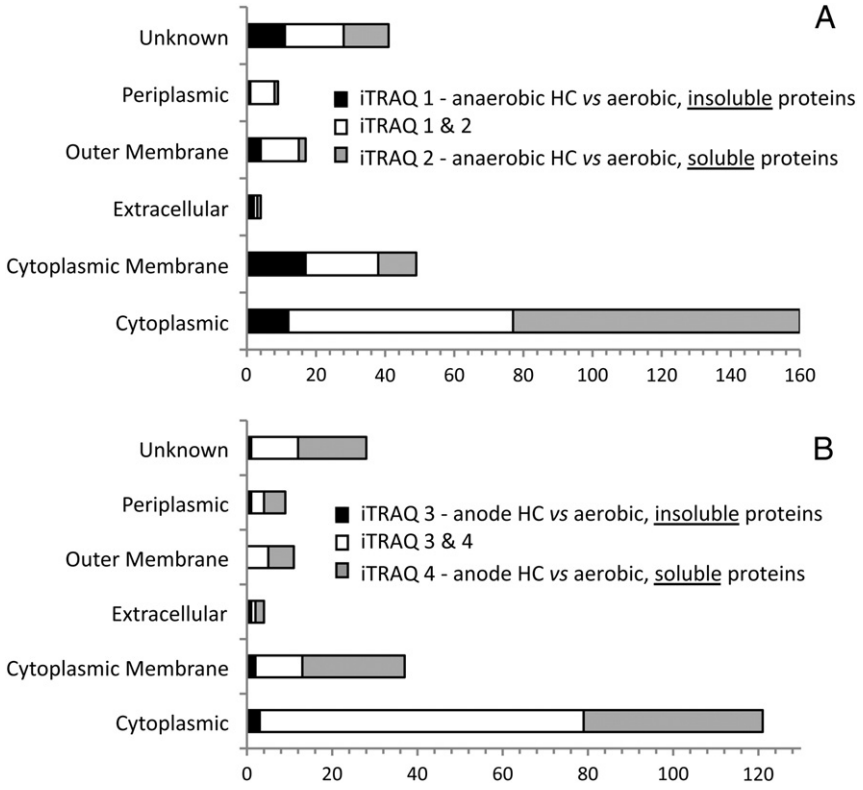

Fig. 1 – Number of proteins identified based on protein localization in: (A) comparison of anaerobic HC vs microaerobic cells in iTRAQ 1 (insoluble) and 2 (soluble); and (B) comparison of anode HC vs microaerobic planktonic cells in iTRAQ 3 (insoluble sub-proteome) and 4 (soluble sub-proteome). Insoluble and soluble sub-proteomes.

reported, and is an issue currently being addressed by the iTRAQ user community [21]. Nonetheless, iTRAQ is a robust technique providing meaningful biological insight into differential protein expression with the additional ease of implementation, replication, and speed as strengths. As discussed in the Materials and Methods, our statistical evaluations and estimation of *p*-values provide confidence that the observed differences in regulation noted below are of biological significance. In Sections 3.3 to 3.8 we discuss selected examples of differential regulation in relation to the microbial physiology of *A. butzleri* growing on electrodes in MFCs and in planktonic culture.

3.3. *Flagellin/biofilm formation*

Flagella play a number of important roles in bacteria, particularly in movement toward or away from nutrients and other solutes (chemotaxis) and in response to oxygen gradients (aerotaxis). They also play a role in biofilm formation and non-motile mutants have been shown to be defective in biofilm formation in many species (e.g. in *E. coli*, *Listeria monocytogenes* [38] and *P. aeruginosa* strains [39]). It is noteworthy that biofilm formation by *Campylobacter jejuni*, an organism closely related to *A. butzleri* ED-1, is flagella-dependent [40], and proteomic analysis of *C. jejuni* biofilms revealed up-regulation of flagella and other 'motility complex' associated proteins compared to planktonic cells [41]. The significant ($p = 5.87 \times 10^{-8}$) 2.3-fold up-regulation of the flagellin protein encoded by ABED_2065 in anode biofilm (anode HC) vs non-HC microaerobic cells (Table 2) was of interest. This is very strong evidence based on 209 peptide quantitations. ABED_2064 and ABED_2065 are adjacent genes encoding the flagellins FlaB and FlaA, which have highly similar protein sequences (91% identity) and are conserved in *A. butzleri* RM4018 (ABU_2254 and ABU_2255 respectively). There has been some confusion regarding the labelling of the two flagellin genes in *Arcobacter* spp. Ho et al. [42] have designated the upstream gene *flaA* and the downstream gene *flaB*, but the genome sequence annotation for RM4018 designates the upstream gene (protein product ABU_2255) as *flaB* and the downstream gene (protein product ABU_2254) as *flaA* (the genes are transcribed in the opposite direction to the genome annotation) [19]. Here, we follow the former convention; hence the anodically-up-regulated ABED_2065 corresponds to FlaA, which is highly expressed and has been shown to be necessary for motility in *Arcobacter* spp. [42]. Consistent with the literature, we find no up-regulation of FlaA between anaerobic HC (planktonic in chamber) vs microaerobic cells, suggesting that up-regulation is anode-specific.

The most likely reasons for FlaA up-regulation at the anode are that it is involved in biofilm formation and/or the flagella play a role in transferring electrons from the cell, across the outer membrane and on to the anode. We postulate that the anode-specific up-regulation of flagella observed in *A. butzleri* ED-1 is at least in part due to their role in biofilm formation.

The polar flagella of strain ED-1 are very long and thin in microaerobic cells (4.3±0.78 μm long by 0.02±0.004 μm), whereas surface-attached cells (anode HC) have flagella of more varying lengths, many of which appear to be flatter and thickened close

Table 1 – Differentially expressed proteins under anaerobic planktonic vs microaerobic planktonic conditions: iTRAQ 1 of insoluble sub-proteome and 2 of soluble sub-proteome.

iTRAQ	Locus tag	Protein description	Functional category [a]	Loc. [b]	Fold change anaerobic planktonic vs microaerobic planktonic [c]	No. peptides	Score	*p*-value
1	ABED_1706	50S ribosomal protein L7/L12	TL	CYT	−2.5	37	6.5	2.84×10^{-7}
1	ABED_1897	Thioredoxin	RS	CYT	−1.9	64	3.1	7.39×10^{-4}
1	ABED_1235	DNA-binding protein HU	NU	UN	−1.9	57	3.9	1.39×10^{-4}
1	ABED_0225	30S ribosomal protein S18	TL	CYT	−1.8	22	3.0	9.34×10^{-4}
1	ABED_1364	Serine hydroxymethyltransferase	TF	CYT	−1.8	30	4.0	1.06×10^{-4}
1	ABED_1194	Argininosuccinate synthase	IM	CYT	−1.8	22	3.2	5.98×10^{-4}
1	ABED_0457	Outer membrane fibronectin-binding protein	CA	OM	−1.6	81	4.1	7.13×10^{-5}
1	ABED_1736	Putative citrate lyase	IM	CYT	−1.6	54	4.2	6.29×10^{-5}
1	ABED_0272	Alkyl hydroperoxide reductase/thiol specific antioxidant	RS	CYT	−1.4	69	3.8	1.48×10^{-4}
1	ABED_1343	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	IM	CYT	−1.3	176	4.1	8.41×10^{-5}
1	ABED_1338	Ni/Fe-hydrogenase, large subunit	ET	CM	1.4	44	3.3	4.57×10^{-4}
1	ABED_0282	Fumarate reductase iron-sulfur subunit	ET/IM	CM	1.6	47	5.4	4.00×10^{-6}
1	ABED_1484	ATP synthase F0 sector, subunit B	ET	UN	1.6	18	2.8	1.65×10^{-3}
1	ABED_1871	Ubiquinol cytochrome c oxidoreductase, cytochrome c1 subunit	ET	PP	1.6	37	4.2	7.02×10^{-5}
1	ABED_1873	Ubiquinol cytochrome c oxidoreductase, 2Fe-2S subunit	ET	CM	1.8	14	2.7	1.83×10^{-3}
1	ABED_0476	Acetate permease	TB	CM	1.9	20	3.6	2.65×10^{-4}
1	ABED_0581	TonB-dependent receptor protein	TB	OM	2.6	22	5.2	5.88×10^{-6}
1	ABED_0582	TonB-dependent receptor protein	TB	OM	2.9	37	7.1	8.15×10^{-8}
1	ABED_1576	Putative cytochrome	ET	CM	3.8	11	3.7	1.90×10^{-4}
1	ABED_1575	Cytochrome c class I	ET	CM	5.3	4	2.3	4.78×10^{-3}
2	ABED_0345	30S ribosomal protein S15	TL	CYT	−3.8	10	2.5	3.05×10^{-3}
2	ABED_0094	50S ribosomal protein L13	TL	CYT	−2.8	11	2.6	2.45×10^{-3}
2	ABED_0663	Oxidoreductase	ET	UN	−2.5	30	3.0	8.92×10^{-4}
2	ABED_1626	ADP-L-glycero-D-manno-heptose-6-epimerase	CA	UN	−2.2	17	2.6	2.58×10^{-3}
2	ABED_0502	Methyl-accepting chemotaxis protein	MC	CM	−2.1	23	3.3	4.57×10^{-4}
2	ABED_1949	DNA-binding ferritin-like protein (Dps/NapA)	RS	CYT	−2.0	155	20.8	1.62×10^{-21}
2	ABED_0457	Outer membrane fibronectin-binding protein	AB	OM	−1.9	185	11.4	3.72×10^{-12}
2	ABED_1897	Thioredoxin	RS	CYT	−1.8	15	2.6	2.31×10^{-3}
2	ABED_1864	Acyl carrier protein, putative	FA	CYT	−1.8	12	3.6	2.41×10^{-4}
2	ABED_1706	50S ribosomal protein L7/L12	TL	CYT	−1.7	71	5.9	1.19×10^{-6}
2	ABED_1980	Sodium:sulfate symporter family protein	TB	CM	−1.6	4	2.1	7.28×10^{-3}
2	ABED_1472	Peptidyl-prolyl cis-trans isomerase	PF	CYT	1.6	18	2.9	1.21×10^{-3}
2	ABED_1871	Ubiquinol cytochrome c oxidoreductase, cytochrome c1 subunit	ET	PP	1.7	48	5.4	3.98×10^{-6}
2	ABED_1045	TonB-dependent receptor	TB	CM	1.9	8	2.1	8.46×10^{-3}
2	ABED_0476	Acetate permease	TB	CM	2.0	19	3.5	3.24×10^{-4}
2	ABED_0474	Sodium:solute symporter family protein	TB	CM	2.2	8	2.6	2.67×10^{-3}
2	ABED_0704	50S ribosomal protein L22	TL	CYT	2.2	3	1.6	2.51×10^{-2}
2	ABED_1623	50S ribosomal protein L28	TB	UN	2.8	3	1.7	1.99×10^{-2}
2	ABED_0581	TonB-dependent receptor protein	TB	OM	3.0	20	5.1	7.54×10^{-6}
2	ABED_0582	TonB-dependent receptor protein	TB	OM	3.4	46	9.4	4.37×10^{-10}
2	ABED_0477	Predicted membrane protein	HY	CM	3.9	8	6.3	4.84×10^{-7}
2	ABED_1576	Putative cytochrome	ET	CM	4.4	8	4.0	9.87×10^{-5}

[a] Functional category: AB — amino acid biosynthesis; CA — cell adhesion; CM — carbohydrate metabolism; ET — energy metabolism and electron transport; FA — fatty acid metabolism; HY — hypothetical; NU — nucleic acid metabolism; MC — motility and chemotaxis; PH — protein folding; IM — intermediary metabolism; RS — response to stress; TB — transport proteins; TF — transferase; TB — transport proteins; TL — translation.
[b] Localization: CYT — cytoplasmic; CM — cytoplasmic membrane; OM — outer membrane; EC — extracellular; PP — periplasmic; and UN — unknown, as indicated by PSORT predictions from the genome website (http://www.psort.org/psortb/).
[c] Fold; fold-change and statistical significance are indicated by the score (−log10 of the *p*-value). The *p*-values (or scores) are included to give an indication of statistical significance.

to the bacterial cell surface (Fig. 3). There appear to be straight shorter filaments (though there is considerable variation with mean dimensions of 0.38±0.14 μm long by 0.08±0.01 μm thick) that interconnect neighbouring cells and also show "nodes" where threads interconnect (Fig. 3C). We suggest that these structures may play a communication role with the anode

Table 2 – Differentially expressed proteins under anaerobic anodic vs microaerobic planktonic conditions: iTRAQ 3 of insoluble sub-proteome and iTRAQ 4 of soluble sub-proteome.

iTRAQ	Locus tag	Protein description	Functional category [a]	Loc. [b]	Fold change Anaerobic planktonic vs microaerobic planktonic [c]	No. Peptides	Score	*p*-value
3	ABED_0094	50S ribosomal protein L13	TL	CYT	−3.2	7	4.9	1.29×10^{-5}
3	ABED_1235	DNA-binding protein HU	RS	UN	−2.5	96	4.5	3.35×10^{-5}
3	ABED_1832	50S ribosomal protein L19	TL	CYT	−1.9	18	3.7	2.24×10^{-4}
3	ABED_0476	Acetate permease	TB	CM	1.6	37	2.1	7.45×10^{-3}
4	ABED_0165	Major facilitator transporter	TB	CM	−4.5	33	5.8	1.76×10^{-6}
4	ABED_0703	30S ribosomal protein S19	TL	CYT	−3.6	4	1.8	1.58×10^{-2}
4	ABED_1884	Cytochrome c oxidase, cbb3-type, subunit III	ET	CM	−3.1	31	5.0	1.07×10^{-5}
4	ABED_0710	50S ribosomal protein L24	TL	CYT	−2.2	28	4.1	8.36×10^{-5}
4	ABED_1886	Cytochrome c oxidase, cbb3-type, subunit II	ET	PP	−2.1	75	7.8	1.44×10^{-8}
4	ABED_1974	Conserved hypothetical protein	HY	EC	−2.1	219	13.0	1.05×10^{-13}
4	ABED_1883	Conserved hypothetical protein	HY	CM	−1.8	19	3.0	1.10×10^{-3}
4	ABED_0241	Pyruvate kinase	IM	CYT	−1.7	18	2.3	5.13×10^{-3}
4	ABED_1484	ATP synthase F0 sector, subunit B	ET	UN	−1.6	56	2.7	1.92×10^{-3}
4	ABED_0282	Fumarate reductase iron-sulfur subunit	ET/IM	CM	−1.5	51	2.5	3.27×10^{-3}
4	ABED_0457	Outer membrane fibronectin-binding protein	ET/IM	OM	−1.4	198	6.0	9.22×10^{-7}
4	ABED_0283	Fumarate reductase flavoprotein subunit	ET/IM	CM	−1.4	119	4.4	3.81×10^{-5}
4	ABED_1482	F0F1 ATP synthase subunit alpha	ET	CYT	−1.2	392	6.9	1.41×10^{-7}
4	ABED_1706	50S ribosomal protein L7/L12	TL	CYT	−1.1	229	1.9	1.41×10^{-2}
4	ABED_1797	30S ribosomal protein S7	TL	CYT	1.3	92	2.1	8.29×10^{-3}
4	ABED_0715	50S ribosomal protein L18	TL	CYT	1.4	32	2.0	1.07×10^{-2}
4	ABED_1864	Acyl carrier protein, putative	FA	CYT	1.5	65	2.2	5.98×10^{-3}
4	ABED_0293	Citrate synthase	IM	CYT	1.6	94	4.5	3.29×10^{-5}
4	ABED_1343	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	IM	CYT	1.6	126	3.6	2.25×10^{-4}
4	ABED_1737	Fumarate hydratase, class I	IM	CYT	1.8	27	1.9	1.38×10^{-2}
4	ABED_0260	Methylcitrate synthase	IM	CYT	1.8	28	2.4	4.05×10^{-3}
4	ABED_1944	Acetolactate synthase	IM	CYT	1.8	35	3.0	8.93×10^{-4}
4	ABED_1986	Hypothetical protein	HY	UN	2.0	16	1.8	1.48×10^{-2}
4	ABED_0272	Alkyl hydroperoxide reductase/thiol specific antioxidant	RS	CYT	2.1	51	5.4	3.95×10^{-6}
4	ABED_1713	Elongation factor Tu	TL	CYT	2.1	125	5.4	3.80×10^{-6}
4	ABED_2065	Flagellin	MC	UN	2.3	209	7.2	5.87×10^{-8}
4	ABED_1964	O-Acetylhomoserine sulfhydrylase	AB	CYT	2.4	10	1.6	2.40×10^{-2}
4	ABED_1364	Serine hydroxymethyltransferase	T	CYT	2.5	17	2.3	4.90×10^{-3}
4	ABED_0259	2-Methylisocitrate lyase	IM	CYT	3.4	14	2.4	3.55×10^{-3}

[a] Functional category: AB — amino acid biosynthesis; CA — cell adhesion; CM — carbohydrate metabolism; ET — Energy metabolism and electron transport; FA — fatty acid metabolism; HY — hypothetical; NU — nucleic acid metabolism; MC — motility and chemotaxis; PH — protein folding; IM — intermediary metabolism; RS — response to stress; TB — transport proteins; TF — transferase; TL — translation.
[b] Localization: CYT — cytoplasmic; CM — cytoplasmic membrane; OM — outer membrane; EC — extracellular; PP — periplasmic; and UN — unknown, as indicated by PSORT predictions from the genome website (http://www.psort.org/psortb/).
[c] Fold; fold-change and statistical significance are indicated by the score (−log10 of the *p*-value). The p-values (or scores) are included to give an indication of statistical significance.

surface for the anode-associated cells, since the structures observed bear some similarities to conductive nanowires observed in electrogenic *Geobacter* spp. [12,43] and electrogenic *Shewanella* spp. [10].

It has been shown that a *G. sulfurreducens* mutant selected for increased current generation at an anode expresses flagella (unlike its parent strain), and has an increased propensity to form biofilms [44]. The lower abundance of ABED_0502 (−2.1 fold in anaerobic HC vs microaerobic cells), a methyl-accepting protein that is a sensor for chemotaxis (82% identity to ABU_0528), perhaps suggesting that the anaerobic bacteria in the half-cell are less motile and therefore that the ABED_2065 flagellin is probably not up-regulated in preparation for a role in flagella movement. Additionally, it is known that *C. jejuni* cells can secrete a number of effector proteins into eukaryotic host cells via the flagellar apparatus; this process requires either flagellin FlaA or FlaB, and an accessory protein CiaB (*Campylobacter* invasion antigen) [45]. *A. butzleri* ED-1 also encodes a CiaB antigen, ABED_1440 with 59% similarity to the CiaB antigen from *C. jejuni* NCTC11168. Further studies are required to determine the role of ABED_2065 and related mechanisms. An additional CLUSTAL comparison between

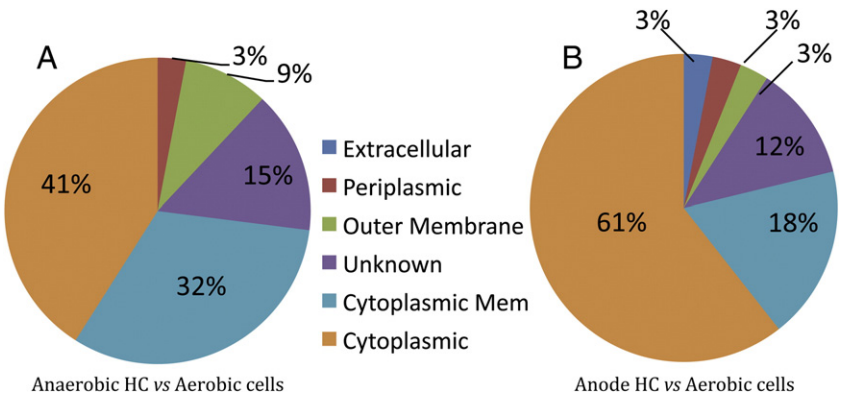

Fig. 2 – Pie charts of localization of regulated proteins based on merged data, from (A) iTRAQ 1 (insoluble sub-proteome) and 2 (soluble sub-proteome), comparison of anaerobic planktonic with microaerobic planktonic cells; and (B) iTRAQ 3 (insoluble sub-proteome) and 4 (soluble sub-proteome), comparison of anodic with microaerobic planktonic cells.

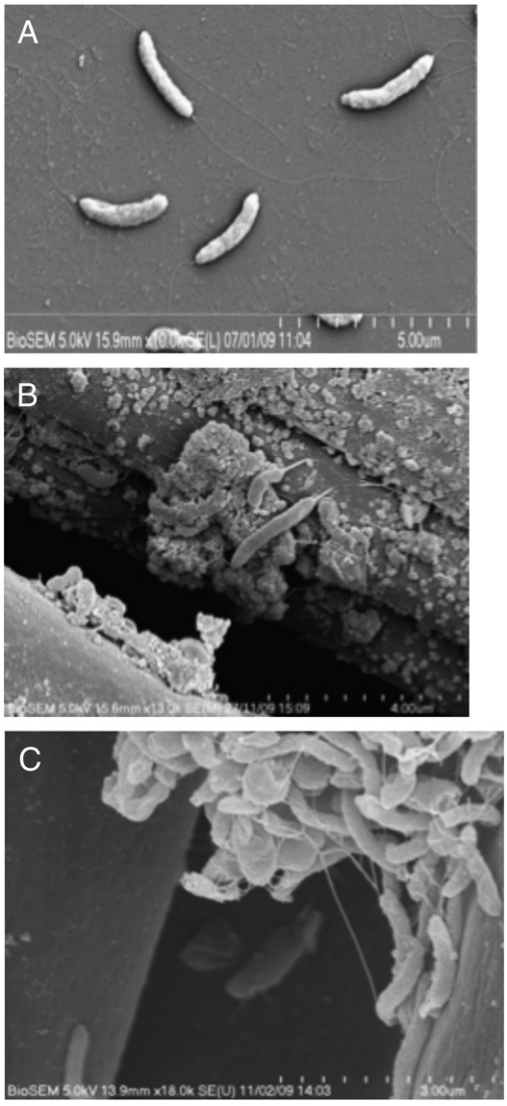

Fig. 3 – SEM images for *A. butzleri* ED-1 as: (A) from microaerobically grown planktonic culture; (B) anodic biofilm in a half-cell; and (C) another view of an anodic biofilm in a half-cell showing "nodes" where threads interconnect. See Supplementary Information 1 for SEM imaging protocol.

ABED_2065 and FliC from *E. coli* and other homologs in other proteobacteria can be found in Supplementary Table 3.

3.4. *Adhesins*

ABED_1974, an uncharacterized protein, was 2.1-fold less abundant in anodic HC vs microaerobic cells. BLAST alignment suggests ABED_1974 is an adhesin gene formed by an integration and gene fusion event: *A. butzleri* RM4018 contains a smaller uncharacterized protein (ABU_2179) homologous to ABED_1974 at the N- and C-termini, but lacks an internal part of the protein that matches instead to hemagglutinin/hemolysin-like proteins from *Pectobacterium carotovorum*. MyHits analysis [46] of ABED_1974 reveals 2 copies of a hemolysin-type calcium-binding repeat, while REPRO analysis [47] indicates that it also contains cadherin-like repeats, and as such may have a possible role in adhesion or biofilm formation. Another putative adhesin ABED_0457 was less abundant in anaerobic *A. butzleri* ED-1 cells, whether anode-associated (1.4-fold, 9.22×10^{-7} *p*-value) or planktonic in the HC chamber (1.9-fold, 3.72×10^{-12} *p*-value). BLAST analysis shows that ABED_0457 is ca. 92% identical to the outer membrane fibronectin-binding protein CadF from *A. butzleri* RM4018 (ABU_0481) and 51% to the OmpA/MotB domain protein precursor from *Arcobacter nitrofigilis* strain ATCC 33309 (ARNIT_0745). The lower abundance of ABED_1974 and ABED_0457 in anode-associated (biofilm) cells suggests that these particular adhesins are not involved in the biofilm formation process that *A. butzleri* ED-1 undergoes during contact with an anode. It may be that adhesins inappropriate for contact with a non-cellular surface are down-regulated under these conditions.

3.5. *Electron transport chain*

Given its initial characterization as an exoelectrogen in an acetate-fed MFC [17], alterations in abundance of ED-1 proteins associated with electron transport under anodic or anaerobic conditions are of interest. Exoelectrogens typically transfer their electrons to a solid terminal electron acceptor via a combination of outer membrane or matrix cytochrome-associated proteins, conductive pili or nanowires, or soluble mediators, with the

potential for functional redundancy within a single species [10,48,49]. To date, the extracellular electron transport mechanisms of *A. butzleri* ED-1 are relatively undefined: it lacks close homologs of membrane-bound multi-haem cytochromes such as OmcS and OmcZ from *G. sulfurreducens* [11] and MtrC from *S. oneidensis* [9], which are known to be involved in this process [18].

Like the type strain RM4018 [19], *A. butzleri* ED-1 encodes protein complexes for microaerobic respiration, such as ubiquinol cytochrome-*c* oxidase, *cbb*$_3$-type cytochrome-c oxidase and F0/F1 ATPase, although it lacks the terminal cytochrome-*bd* oxidase of RM4018 (Fig. 4) [18]. It also encodes anaerobic respiration activities such as fumarate reductase, nitrate reductase, nitrite reductase, nitric oxide reductase and a putative trimethylamine oxide reductase. Cytochrome *cbb*$_3$ oxidases are a diverged group of proteobacterial oxidases with a very high affinity for oxygen, which are a specialized adaptation to microaerobic environments [50].

Subunits II and III of the ED-1 *cbb*$_3$-type oxidase (ABED_1886 and 1884, respectively) were down-regulated (2.1- and 3.1-fold, respectively) in anode HC compared to non-HC microaerobic cells (Fig. 4; Table 2), consistent with reduced use of oxygen as a terminal electron acceptor under these conditions. In contrast, two subunits of the ubiquinol cytochrome-*c* oxidase (ABED_1871 and 1873) were up-regulated (1.7-fold/3.98×10^{-6} *p*-value and 1.8-fold/1.83×10^{-3} *p*-value, respectively) under anaerobic HC compared to microaerobic conditions (Table 1), while the fumarate reductase Fe-S and flavoprotein subunits (ABED_0282 and 0283) were statistically down-regulated (1.5-fold/3.27×10^{-3} *p*-value and 1.4-fold/3.81×10^{-5} *p*-value, respectively) under anodic (biofilm) conditions (Table 2). The latter effect may be due to the use of the electrode as an electron acceptor instead of routing electron flow through the fumarate reductase pathway, and also due to changes in the fluxes through the TCA cycle (see Section 3.5). In contrast, ABED_0282 was up-regulated 1.6-fold in anaerobic HC cells compared to microaerobic condition (Table 1), while ABED_0283 abundance was not significantly altered under the same conditions (Supplementary Table 4). This suggests a specific difference in electron flow between the anode-attached and planktonic cells in the half-cell.

As well as the various cytochrome-containing complexes described above, ED-1 also encodes a number of poorly-characterized potential cytochromes including putative cytochrome-*c* peroxidases (ABED_0838, 1583 and 1935), a novel *b*-type cytochrome (ABED_2146) and a number of putative *c*-type cytochromes (ABED_0851, 1229, 1575, 1576, 1583, 1729, and 2145). Interestingly, ABED_1575 and 1576 were up-regulated (5.3- and 4.4-fold, respectively) under anaerobic half-cell conditions (Table 1; Fig. 4) compared to microaerobic conditions. The genes encoding these cytochromes form part of a potential 6-gene operon, also including a transcriptional regulator (ABED_1577) and putative thioredoxin, Fe-S and permease proteins (ABED_1574-1572, respectively) [18]. The data suggest these anaerobically-induced cytochromes may have a role in anaerobic electron transport by acetate-fed *A. butzleri* ED-1, but further studies are needed.

3.6. *Methylcitrate cycle and citramalate cycle*

Six proteins up-regulated in *A. butzleri* ED-1 harvested from the anodic cells play a role in the methylcitrate cycle, which suggests a potential role for this cycle in the electrogenic processes of *A. butzleri* ED-1 (Fig. 5). The up-regulation of the oxaloacetate or pyruvate generating enzymes in the anode-

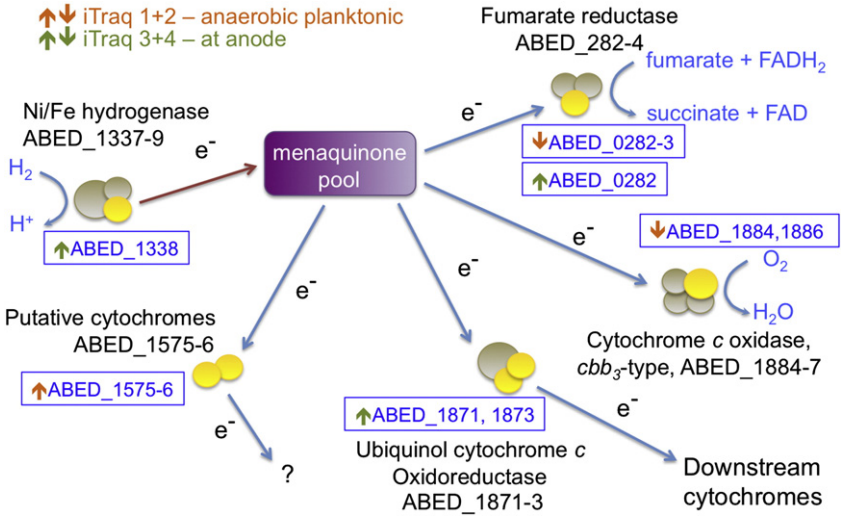

Fig. 4 – Diagram of components of the electron transport chain that are differentially expressed in ED-1, and their relationship to the menaquinone pool. Subunits of the relevant protein complexes are in grey (non-cytochrome subunits) or yellow (cytochrome subunits). Enzyme complex names and the corresponding protein subunit IDs are shown in black, with those subunits negatively or positively differentially regulated in anode HC or anaerobic HC cells shown with red or green (↑↓) down and up arrows, respectively, from Table 1 to 2. Chemical reactions catalyzed by the complexes are shown where applicable. Electron flow is indicated by arrows.

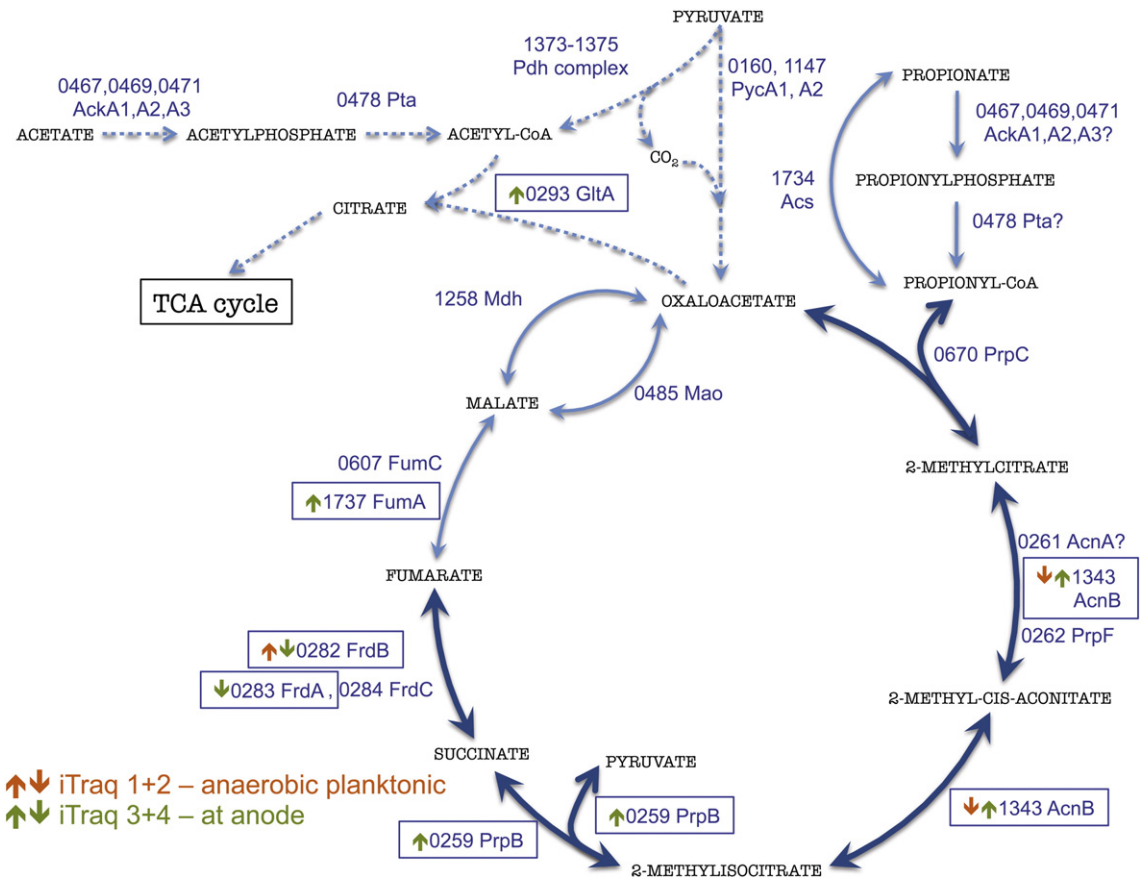

Fig. 5 – Proposed methylcitrate cycle of *A. butzleri* ED-1. The genes of the enzymes are indicated (all are ABED_ genes) along with the gene abbreviations. The proteins in boxes and with arrows (↑↓) denote positively or negatively differentially regulated genes, respectively, during anode HC or anaerobic HC growth compared to microaerobic conditions from Table 1 to 2. The dashed arrows indicate compounds entering or exiting the cycle. Thicker arrows indicate pathways up-regulated during anode HC growth vs microaerobic conditions from Table 2. Details of the encoding genes can be found in Supplementary Information 1.

associated cells vs microaerobically grown non-half-cell cells may be due to increased amounts of acetate supplied to the bacteria cultivated in the half-cells. These proteins are methylcitrate synthase (ABED_0260, 1.8-fold), bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase (ABED_1343, 1.6-fold), and 2-methylisocitrate lyase (ABED_0259, 3.4 fold). There is no up-regulation of these enzymes in cells harvested from the anaerobic planktonic phase of the half-cells vs microaerobically grown cells, which were subject to the same levels of acetate as the cells harvested from the anodic biofilm, but do not have a readily-available electron acceptor for metabolism. Furthermore, one of these proteins, bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase (ABED_1343) was 1.3-fold down-regulated (8.41×10^{-5} *p*-value) in the anaerobic HC cells vs microaerobically grown cells. It may be that the citramalate cycle is important in acetate utilization by *A. butzleri* ED-1 as it generates the oxaloacetate, which serves as a counterpart to the acetyl-CoA, generated from acetate and therefore up-regulation of acetate metabolic enzymes of anodic cells is unsurprising. One can speculate that these enzymes also have a biofilm role, as acetate metabolism is thought to be involved in the biofilm formation of *C. jejuni* [40], although whether this is the case in *A. butzleri* ED-1 is an open question.

3.7. *TonB-receptors*

iTRAQ data show that TonB-dependent receptor proteins ABED_0581 and ABED_0582 were more abundant (3.0- and 3.4-fold, respectively) in anaerobic HC cells compared to non-half-cell microaerobic cells (Table 1). A concomitant 1.78-fold increase in abundance of ABED_1478 encoding biopolymer transport protein ExbB, a component of the Ton system, was observed under the same condition (Supplementary Table 4). The TonB–ExbB–ExbD system is found widely in Gram-negative bacteria and is used for transport of solutes such as ferric siderophores, vitamin B12, and group B colicins from the outer membrane to the cytoplasm [51]. A variety of TonB dependent receptor (TBDR) proteins interact with the TonB–ExbB–ExbD complex: the genome sequence indicates that *A. butzleri* ED-1 has 17 TBDR proteins and two TonB–ExbB–ExbD systems in clusters (ABED_0653-0655 and ABED_1476-1478) [18]. The TBDR proteins bind to the TonB protein and mediate conformational changes in the protein, which when closed has an N-terminal plug capping the gated channel. The complex was originally shown to transport iron chelates such as ferrienterobactin and also vitamin B_{12}. However, the range of substrates is now known to be much wider [52].

A third TBDR protein, ABED_1045, was 1.9-fold more abundant in anaerobic HC cells relative to non-HC microaerobic cells. ABED_1045 from *A. butzleri* ED-1 has no equivalent in *A. butzleri* RM4018, but has a homolog with low similarity (51% identical) but conserved gene order in the non-electrogenic strain *Arcobacter*-L [18], suggesting that ABED_1045 is more likely to be involved in the reaction of *A. butzleri* ED-1 to its environment than in its electrogenic properties.

3.8. *The sodium-solute symporter family*

The iTRAQ data show that proteins similar to the sodium-solute symporter family differ in abundance between microaerobic and anaerobic HC conditions. The generalized transport reaction catalyzed by the members of this family is: solute (out)+nNa^+ (out)→solute (in)+nNa^+ (in). Three such proteins, ABED_0474 (2.2-fold), ABED_0476 (2.0-fold) and ABED_0477 (3.9-fold), were up-regulated in anaerobic HC conditions. ABED_0476 was also up-regulated (1.6-fold) in the anode-associated bacteria, while ABED_1980 was down-regulated (1.6-fold) in anaerobic HC cells. The genes for ABED_0474 (a putative acetate permease) and ABED_0475 (a small inner-membrane protein of unknown function) are next to and code for highly similar proteins to ABED_0476 (68%) and ABED_0477 (63%).

A possible role of these proteins raised by a BLAST analysis against the proteomes of *E. coli* strains shows that the closest fits (~60% identity) for ABED_0474 and ABED_0476 are several ActP cation/acetate symporters implicated in acetate transport [53]. Some of the identities of protein sequences from *A. butzleri* ED-1 versus those from other proteins (BLAST analyses) are shown in Supplementary Table 5. The expression of the operon that contains ActP is up-regulated in acetate-limited *E. coli* [54] and *G. sulfurreducens* [55] and the increase in abundance of ABED_0474, ABED_0476 and ABED_0477 proteins in acetate-limited anaerobic conditions suggests that they too form an acetate permease system. In other studies, ActP has been shown to facilitate the resistance to toxic tellurite in *R. capsulatus* [56], while in *E. coli*, biofilm formation is linked to the differential expression of the *actP* gene [57]. In *G. sulfurreducens*, a similar gene arrangement is found where 3 of the 4 acetate permeases are adjacent to a small membrane protein in an operon-like structure [55]. This leads us to suggest that the genes encoding ABED_0476 and ABED_0477 are functionally associated in acetate transport and that the genes for ABED_0474 and ABED_0475 arose by gene duplication.

Another interesting observation is for ABED_1980, also a member of the sodium-sulfate symporter family, which was less abundant under the same acetate-limited anaerobic conditions. A BLAST analysis reveals that ABED_1980 may be a dicarboxylate acid antiporter. In *E. coli*, such transporters exchange dicarboxylate and tricarboxylate ions such as tartrate/succinate, citrate/succinate and 2-oxoglutarate/malate and have a defined role in anaerobic respiration with fumarate. The closest homolog of ABED_1980 in *E. coli* is TtdT, a tartrate/succinate exchanger with 46% homology, but it is also related to the CitT protein, a citrate/succinate antiporter (38% identity). No equivalent proteins are found in either *G. sulfureducens* or *S. oneidensis*.

4. Conclusions

Here, we employed iTRAQ to investigate changes in the soluble and insoluble sub-proteomes of a functioning exoelectrogen. We identified 75 differentially expressed proteins in the Epsilonproteobacterium *A. butzleri* ED-1 during anaerobic growth on an insoluble electrode in a half-cell. Interestingly, the results emphasize the involvement of FlaA, a flagellin, in the anodic cells while generating a negative anode potential. This suggests that FlaA may play a role in anode-cell communication related to biofilm formation and electrogenicity; we also observed filaments resembling pili providing another example of an exoelectrogen that might utilize pili or so-called “nanowires” for current generation. Furthermore, the analysis suggested the involvement of two novel cytochromes in anaerobic electron transport, as well as a role of the methylcitrate cycle for the metabolism of anodic *A. butzleri* ED-1. Future mutational inactivation and other functional investigations, will allow us to discern further the characteristics of these and other identified proteins and their mechanisms. Nonetheless, these results highlight the potential of proteomics to identify novel proteins of relevance to the exoelectrogenic process. A better understanding of these organisms will lead us to improve their application to bioenergy production.

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Supplementary Information Available: Additional supplementary information and the comprehensive list of proteins identified in all experiments, with pertinent statistics in Excel Spreadsheet format.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.09.039.

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